(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 702 085 A1

(12) EUROPEAN PATENT APPLICATION

(43) Date of publication:
20.03.1996 Bulletin 1996/12

(21) Application number: **95201936.2**

(22) Date of filing: **14.07.1995**

(51) Int. Cl.[6]: **C12N 15/86**, C12N 15/47, C07K 14/145, A61K 39/205, C07K 14/16, C07K 14/185

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL PT SE

(30) Priority: **18.07.1994 EP 94202089**

(71) Applicant: **Akzo Nobel N.V.**
NL-6824 BM Arnhem (NL)

(72) Inventor: **Conzelmann, Karl Klaus**
D-72406 Bisengen (DE)

(74) Representative: **Mestrom, Joannes Jozef Louis et al**
Patent Department Pharma
N.V. Organon
P.O. Box 20
Weth. van Eschstraat 1
NL-5340 BH Oss (NL)

(54) Recombinant infectious non-segmented negative strand RNA virus

(57) The present invention provides the generation of infectious replicating non-segmented negative-stranded RNA virus, entirely from cloned cDNA.

This process offers the possibility to introduce mutations into the virus genome by means of recombinant DNA techniques.

EP 0 702 085 A1

Description

The present invention is concerned with a genetically manipulated infectious replicating non-segmented negative-stranded RNA virus mutant and a process for the preparation of such a mutant.

Rabies virus (RV) is an example of a non-segmented negative-stranded RNA virus of the Rhabdoviridae family. Other species belonging to this family are vesicular stomatitis virus (VSV), infectious hematopoietic necrosis virus (IHNV) viral haemorrhagic septicaemia virus (VHS, Egtved virus), bovine ephemeral fever virus (BEFV), and sonchus yellow net virus (SYNV).

Beside the family of Rhabdoviridae also viruses belonging to the Paramyxoviridae (e.g. sendai virus (SV), para-influenza virus (PIV) type 2 and 3, Newcastle disease virus (NDV), mumps virus (MUV), measles virus (MEV) and canine distemper virus (CDV)) and Filoviridae, and several viruses not assigned to a family (e.g. Borna disease virus; BDV) have a non-segmented negative-stranded RNA genome.

The overall genomic organisation in the non-segmented negative-stranded RNA viruses of the various families is comparable. Especially between the paramyxoviridae and the rhabdoviridae, there are only minor differences in the overall genomic organisation (Tordo et al., Seminars in Virology 3: 341-357, 1992).

RV can infect all warm-blooded animals, and in nearly all instances after establishment of symptoms the infection ends in death. Dog rabies is still important in many parts of the world: infected dogs cause most of the estimated 75,000 human rabies cases that occur each year world-wide. In many countries of Europe, and in the United States and Canada, wildlife rabies has been increasing in importance.

The clinical features of rabies are similar in most species, but there is great variation between individuals. Following the bite of a rabid animal the incubation period is usually between 14 and 90 days, but may be considerably longer, and incubation periods of over a year have been documented. Two clinical forms of the disease are recognized furious and dumb or paralytic. In the furious form, the animal becomes restless, nervous, aggressive, and often dangerous as it loses all fear of humans and bites at anything that gains its attention. The animal often cannot swallow, giving rise to the synonym for the disease, "hydrophobia". There is often excessive salivation, exaggerated responses to light and sound, and hyperesthesia. As the encephalitis progresses, fury gives way to paralysis, and the animal manifests the same clinical features as seen throughout in the dumb form of the disease. Terminally, there are often convulsive seizures, coma, and respiratory arrest, with death occurring 2-7 days after the onset of clinical signs.

Rabies virus enters the body in the bite or occasionally the scratch of a rabid animal, or when virus-loaded saliva from a rabid animal enters an open wound. Viral replication in the bite site, in muscle, is followed by invasion of peripheral nerve endings and central movement of viral genome in the cytoplasm of axons to the central nervous system. Viral entry into the spinal cord and then the brain (particularly the limbic system) is associated with clinical signs of neuronal dysfunction. Usually, at about the same time that central nervous system infection causes fury, virions are also shed from the apical end of mucus-secreting cells in the salivary glands and are delivered in high concentrations into saliva.

Throughout the course of rabies, host inflammatory and specific immune responses are only minimally stimulated; the most likely reasons for this are because the infection is non-cytopathic in muscle and in nerve cells and because the infection is largely concentrated in the immunologically sequestered environment of the nervous system.

RV virions like all Rhabdoviruses are composed of two major structural components: a nucleocapsid or ribonucleoprotein (RNP) core and an envelope in the form of a bilayer membrane surrounding the RNP core. The infectious component of all Rhabdoviruses is the RNP core. The genomic RNA is of negative sense and thus cannot serve as a messenger but requires its own endogenous RNA polymerase for transcription of mRNA. The RNA genome is encapsidated by the nucleocapsid (N) protein in combination with two minor proteins, i.e. RNA-dependent RNA polymerase (L) and phosphoprotein (P) to form the RNP core. The membrane component contains two proteins: an trans-membrane glycoprotein (G) and a matrix (M) protein located at the inner side of the membrane. The G-protein is responsible for cell attachment and membrane fusion in RV, and additionally is the main target for the host immune system.

During transcription, the genome directs the sequential synthesis of a short leader RNA and five monocistronic, capped and polyadenylated mRNAs. During replication, the conditional transcription stop and start signals between the cistrons are ignored by the viral polymerase. For both the transcriptase and the replicase reaction the presence of the N-protein complexed with the RNA genome as well as the L- and P-proteins are required. The gene order on the RV genome has been determined and is 3'-leader-N-P-M-G-L-5' as shown in Fig. 1. Each of the mRNAs of RV is translated immediately after transcription. Two events occur sequentially during replication: first the production of an encapsidated complete positive strand RNA complementary to the genome, followed by the production of complete negative-stranded RNA which is also encapsidated by the N, L and P proteins. Finally, the newly assembled RNP cores associate with M-protein and G-protein during the assembly and budding process leading to the release of fully formed and infectious RV virions.

The 11.9 kb genomic RV RNA contains five open reading frames (ORFs) coding for the N, P, M, G and L proteins, in addition to the presence of a pseudogene region (ψ) between the G and L genes (Fig. 1).

Current vaccines for non-segmented negative strand RNA viruses comprise chemically inactivated

virus vaccines or modified live virus vaccines comprising an attenuated virus strain the pathogenicity of which is decreased by multiple passages in cell culture. Chemically inactivated rabies vaccines are e.g.: Rabivac, Behringwerke (human), HDC, Rhone-Poulenc (human), Bayovac-LT, Bayer (vet), Madivac, Hoechst (vet), Epivax-LT, Pitman-Moore, Rabisin, Rhone-Merieux. For RV examples of such attenuated viruses are the vaccine strains SAD B19 and ERA. Inactivated vaccines generally induce only a low level of immunity, requiring repeated immunizations. Furthermore, the neutralization inducing antigenic determinants of the pathogens may become altered by the inactivation treatment, decreasing the protective potency of the vaccine.

In general, attenuated live virus vaccines are preferred because they evoke an immune response often based on both humoral and cellular reactions. However, during cell culture passaging uncontrolled mutations may be introduced into the viral genome, resulting in a population of virus particles heterogeneous with regard to virulence and immunizing properties. Over attenuation during passage in cell culture can also be a problem with these vaccines. One must achieve a delicate balance between ensuring that the vaccine is not virulent while making certain that it is still protective. In addition it is well known that such traditional attenuated live virus vaccines can revert to virulence resulting in disease outbreaks in inoculated animals and the possible spread of the pathogen to other animals.

Moreover, a problem with combined live viral vaccines is the mutual influence of the antigenic components resulting in a decrease of the potency of one or more of the constituting components.

Furthermore, with currently administered live attenuated or inactivated RV vaccines it is not possible to determine whether a specific animal is a carrier of RV field virus or whether the animal was vaccinated. Hence, it can be important to be able to discriminate between animals vaccinated with a RV vaccine and those infected with a field virus so as to be able to take appropriate measures to reduce spreading of a virulent field virus. The introduction of for example a serologically identifiable marker can be achieved by introducing a mutation in a gene encoding a (glyco-) protein of RV which normally give rise to the production of antibodies in an infected host animal.

It is desired to introduce a mutation into the RV RNA genome in a controlled manner such that for example the resulting mutant RV is attenuated or comprises a heterologous nucleic acid sequence encoding epitopes of foreign proteins, e.g. immunological marker proteins or antigens of pathogens. Recombinant DNA techniques are already widely used for this purpose with DNA viruses and positive strand RNA viruses. Examples for recombinant DNA viruses: Aujeszky virus (PRV); Adenoviruses; Vaccinia viruses. Examples for recombinant positive-strand RNA viruses: Alphaviruses (Sindbis V., Semliki forest virus: H.V. Huang, C.M. Rice, C. Xiong, S. Schlesinger (1989) RNA viruses as gene expression vectors. Virus Genes 3, 85-91). Picornaviruses (Polio virus, Hepatitis A-virus, Foot- and mouth-disease virus: J.W. Almond and K.L. Burke (1990) Poliovirus as a vector for the presentation of foreign antigens. Semin. Virol. 1, 11-20). Directed genetic manipulation of RNA virus genomes depends on the ability to produce recombinant RNAs which are accepted as a template by the particular RNA-dependent RNA polymerases. Transcripts generated by many standard DNA-dependent RNA polymerases (e.g. T7 RNA polymerase or cellular RNA polymerase II) and mimicking viral genomes are recognized by the polymerases of many positive stranded RNA viruses. This allowed recovery of infectious viruses or replicons from cDNA transcripts and the application of recombinant DNA technology to manipulate these genomes in a site specific manner. Since RNAs corresponding to the genomes of positive stranded RNA viruses may function as mRNA for translation of the viral polymerases, an infectious cycle may be initiated by introduction of the genome analogs into a cell. The template of the polymerases of negative-stranded RNA viruses, however, exclusively is the RNP complex. Moreover, and in contrast to positive stranded RNA viruses, their genomic or antigenomic RNA may not function as mRNA and thus all viral proteins involved in replication and transcription of artificial RNAs have to be provided in trans.

An appropriate system for encapsidation of genomic RNA analogs of a negative-stranded RNA viruses with a segmented genome in order to provide the appropriate template is recently disclosed by Palese, P. et al., (WO 91/03552). RNA transcripts from influenza virus genome segments were encapsidated by purified proteins in vitro which can be used to transfect cells together with a helper virus. However, it was found that this approach was not successful with RV, a virus having a non-segmented genome. Short model genomes of VSV and RV lacking the major part of the RNA genome comprising the genes encoding the viral proteins could be encapsidated and expressed by plasmid encoded proteins (Pattnaik, A.K. et al, Cell 69, 1011-1020, 1992; Conzelmann, K-K. and M. Schnell, J. Virology 68, 713-719, 1994). This approach involved the co-expression of both the genome analogs optionally comprising reporter gene inserts, and particular viral proteins from transfected plasmids in order to produce defective virus particles. Ballart et al. described a method to obtain infectious measles virus, also a non-segmented negative-stranded RNA virus, from cloned cDNA (The EMBO Journal, 9: 379-384 (1990)). A European Patent Application relating to this method was filed with the author as one of the inventors.

Both the paper and the Application were withdrawn however, since further research revealed that all supposed recombinant viruses were no recombinants at all, but mere progeny virus of the originally used vaccine strain.

Thus it must be concluded, that attempts to obtain infectious recombinant negative-stranded RNA viruses with a large, non-segmented genome which necessi-

tates manipulation of the entire genomes, have failed until now.

The present invention provides a genetically manipulated infectious replicating non-segmented negative-stranded RNA virus mutant, obtainable by recombinant DNA techniques, comprising an insertion and/or deletion in an ORF, pseudogene region or non-coding region of the RV genome.

More specifically the invention provides non-segmented negative-stranded RNA viruses of the paramyxo- and rhabdovirus family.

As explained above, there is a large homology in genomic organisation between the non-segmented negative-stranded RNA virus families. Where the function of encoded proteins in the process of replication, assembly, cell attachment or cell fusion is comparable, these proteins will be referred to further as "analogs". It may be that the function of e.g. two proteins of one family is united in one protein in another family. This is e.g. the case with the F and HN proteins of the paramyxoviridae, that together have the same function as glycoprotein G of the Rhabdoviridae. In this case, the two proteins of the one family will be considered analogons of the one protein of the other family.

The insertion and deletion of one or more nucleic acid residues can be introduced in the RV genome by incorporating the appropriate mutations into the corresponding viral ORF, pseudogene region or non-coding region. This alteration is understood to be a change of the genetic information in the RV ORF or pseudogene of a parent RV thereby obtaining the insertion or deletion RV mutant according to the invention.

A mutation, in which one or more nucleotides are replaced by other nucleotides, a socalled substitution replacement is considered to be the result of a combined deletion and insertion action. This kind of mutation is therefore also considered to be included in the wording: deletion and(/or) insertion.

It is clear that any mutation as defined herein comprises an alteration of appropriate RV sequences such that the resulting RV mutant is still infectious and replicating, i.e. the mutant RV is capable to infect susceptible cells and its mutant RNA genome is capable of autonomously replication and transcription, i.e. no co-expression of RV N, P and L proteins is required.

It goes without saying, that also comprised in the present invention are mutant RVs capable of only one single round of infection, followed by replication (Vide infra).

The genomic organisation of different RV strains is identical. The nucleotide sequence and deduced amino acid sequence analysis of the vaccine strain SAD B19 and the virulent strain PV have been determined (Conzelmann et al., Virology 175, 485-499, 1990 and Tordo et al., Nucleic Acids Res. 14, 2671-2683, 1986; Proc. Natl. Acad. Sci USA 83, 3914-3918, 1986; Virology 165, 565-567, 1988). In Conzelmann et al., 1990 (supra) it is determined that the viral genome of the SAD B19 strain comprises 11.928 nucleotides and that the deduced amino acid sequence of the five viral proteins N, P, M, G and L are highly similar to those of the pathogenic PV strain. The location of the respective ORFs, pseudogene region and intergenic non-coding regions in RV have been determined therein: the coding region of the RV N, P, M, G and L genes correspond with positions 71-1423, 1514-2407, 2496-3104, 3317-4891, 5414-11797, respectively. The pseudogene region (ψ) maps at position 4961-5359, whereas the intergenic regions separating the five cistrons and which are flanked by non-coding sequences containing transcriptional start and stop/poly-adenylation signals map to positions 1483-1484; 2476-2480; 3285-3289; 5360-5383. Although the numbering and the nucleotide sequence of the ORFs, pseudogene region or non-coding regions of the parent RV strain used herein to introduce a mutation is not necessarily the same as that of the SAD B19 or PV strain, the above-mentioned characterisations of these regions exactly define the localisation thereof on the genome of any RV strain.

A method to obtain an attenuated RV from a virulent parental RV strain is to introduce the insertion and/or deletion in an ORF encoding a viral protein, for example such that the activity of the viral protein for host cell attachment and membrane fusion is modified, e.g. reduced. It is known for RV that changes in the amino acid sequence of the trans-membrane glycoprotein G have significant effects on the pathogenicity of the RV. In addition, with regard to attenuation also changes in the matrix (M) protein may influence the conformation of the G protein resulting in an attenuation of the virus. Therefore, mutant RV comprising a deletion or insertion in the ORF encoding the G or M protein are particularly preferred herein.

Also comprised in the present invention are infectious replicating rabies virus mutants capable of only one single round of infection, followed by replication. The advantage thereof is explained below:

Although generally spoken recombinant live vaccines have been proven to be safe and efficacious, there is a risk that the vaccine viruses spread to other animals which are more susceptible for the virus.

Therefore, there is a strong reluctance on both political, ethical and partially scientific grounds, to allow the use of recombinant viruses in the field.

In particular, for risk assessment studies by regulatory authorities with respect to genetically modified vaccine viruses, especially live viruses expressing foreign genes, the aspect of possible shedding of these viruses in the environment is a very important aspect.

Thus, it can be appreciated that rabies virus vaccines which display all the advantages of live virus vaccines but which are confined to the vaccinated animals and are not shedded, are highly desirable.

Such viruses can be made by e.g. mutation of the M-gene, encoding the M(atrix-)protein. The M-protein plays a main role in the assembly of the virus, whereas it additionally influences the incorporation and conformation of the glycoprotein G.

When $M^{(-)}$mutants, lacking a functional M-protein, are grown in manipulated cells that produce the M-protein in trans, intact virus particles are made, that behave like wild-type virus as far as their infectious character towards their natural host is concerned. Once they have infected a host cell however, there is no possibility to form new infectious viruses, since they lack the genetic information to synthesize the M-protein.

Therefore, they remain contained in the host. The advantages of such viruses will be discussed below.

Therefore, in a preferred embodiment the present invention relates to an insertion and/or deletion in the open reading frame encoding the matrix protein M, such that it results in a non-functional matrix protein M, or even in the absence of matrix protein M. The $M^{(-)}$mutant viruses with the non-functional or absent matrix protein M have to be grown in cells that provide a matrix protein M analog in trans, in order to phenotypically complement the virus.

Alternatively, such viruses can be made by e.g. mutation of the G-gene. The G-protein plays a main role early in infection, in the process of cell attachment and membrane fusion, as mentioned before.

It is possible to mutate the G-gene by insertion and/or deletion (or even by deletion of the whole G-gene) to such an extend that the resulting G^- mutant virus is no longer capable of successfully infecting other cells, due to heavily impaired (or even absent) glycoprotein G. Such mutants will further be referred to as G-minus (G^-) mutants.

This kind of mutations of the G-gene is therefore more severe than the mutations described before, that only lead to decreased virulence: real G^- mutants are not infectious, since they lack a functional glycoprotein G.

If such G^-mutant viruses are grown in recombinant host cells complementing for the G-protein, progeny viruses are excreted that are phenotypically G-positive, but genotypically G-negative.

These viruses have an important advantage over G-positive viruses: on the one hand, they are capable of infecting non-complementing host cells, since they possess the G-protein in their membrane. In the infected cells, the G^- mutant viruses replicate as wild-type viruses. This has the advantage that the whole viral genome, including heterologous genes cloned into the recombinant virus, is multiplied, and the encoded genome products will be expressed and processed as with wild-type virus.

On the other hand however, no infectious progeny virus can be made in the host, since normal host cells do not synthesize G-protein, and the mutant virus itself is genotypically G-negative.

Thus, animals infected with G^- mutant virus do not shed infectious virus in the environment. This makes G^- mutants (as well as the $M^{(-)}$ mutants discussed above) very safe as a basis for vaccines.

Alternatively, the G^- mutants according to the invention can be complemented phenotypically by other, non-rabies-, glycoproteins known to play a role in cell attachment.

Since glycoprotein(s) protruding from the viral membrane into the environment are known to determine the cell-specificity, it therefore is possible to target the recombinant infectious rabies virus mutant to specific cells other than the natural host cells of rabies, by chosing the right complementing glycoprotein.

These glycoproteins will further be called "glycoprotein G analogs", to indicate that they are involved in cell-specific attachment, like glycoprotein G.

It should be noticed, that in some viruses, the "glycoprotein G analogs" determining the cell specificity are not glycoproteins but non-glycosylated proteins. It is clear, that these proteins are also within the scope of the invention.

Therefore, in another preferred embodiment of the present invention, the insertion and/or deletion in the open reading frame encoding the glycoprotein G is such that it results in a non-functional glycoprotein G, or even in the absence of glycoprotein G. The $G^{(-)}$mutant viruses with the non-functional or absent glycoprotein G have to be grown in cells that provide a glycoprotein G analog in trans, in order to phenotypically complement the virus.

In an even more preferred embodiment of the present invention, the glycoprotein analog used for complementation is the rabies virus glycoprotein G itself.

Recombinant infectious rabies viruses with a glycoprotein G analog have several important advantages:

a) they can be specifically targeted to certain cells, organs or hosts, depending on the target of the glycoprotein G analog that was chosen,

This implicates that e.g. specifically the respiratory tract or the digestive tract can be targeted. Thus, e.g. mucosal responses can be obtained at a predetermined site.

Alternatively, specific cells of the immune system can be targeted.

b) they can additionally be carriers of foreign genetic information encoding epitopes from non-rabies pathogens as explained above.

Alternatively, they can be carriers of foreign genetic information encoding toxic substances.

A very important application of viruses according to the invention is obtained with viruses having both a glycoprotein G analog according to a) and foreign genetic information according to b).

Recombinant infectious rabies viruses can be obtained according to the present invention, that are targeted to a specific cell type, normally attacked by a non-rabies virus, while at the same time carrying an immunoprotective determinant of that non-rabies virus.

Such a virus induces immunity in the host against the non-rabies virus, whereas at the same time it is fully

safe, due to the lack of genetic information for the glycoprotein G analog.

Another important embodiment of the present invention are viruses according to the present invention that are e.g. targeted to CD4-cells, that represent target cells of HIV, through genotypical complementation with HIV gp120, and that facultatively encode a cytotoxic protein.

Such viruses will selectively attack CD4-cells, and once inside these the cells, they will kill them.

Alternatively, recombinant infectious rabies viruses according to the present invention can provide very safe vaccines against virulent/pathogenic viruses against which at this moment no safe live vaccines exist: a recombinant infectious rabies virus targeted against e.g. the natural target cells of Bovine Respiratory Syncytial Virus (BRSV) through complementation with BRSV glycoprotein G analog, and expressing immunoprotective epitopes of BRSV, gives a very safe vaccine against this disease.

Parainfluenza virus vaccines have so far faced the same problems as BRSV-vaccines. Therefore, recombinant infectious rabies virus with parainfluenza glycoprotein G analog and additional immunogenic epitopes of parainfluenza provides a good and safe vaccine against this disease.

Other important veterinary vaccines based on recombinant infectious rabies virus are made by introduction into the recombinant rabies virus of immunogenic determinants of:

i) the toroviruses; equine, bovine and porcine torovirus,

ii) the coronaviruses; bovine, canine, porcine and feline coronavirus, especially the spike-proteins thereof.

Therefore, a most preferred embodiment of the present invention relates to recombinant infectious rabies virus glycoprotein $G^{(-)}$mutants, complemented with a glycoprotein G analog, and carrying a heterologous nucleic acid sequence encoding an epitope or polypeptide of a pathogenic virus or microorganism.

Alternatively, attenuation of the RV may be obtained by altering the enzyme activity of the RV replicase or transcriptase so that the enzyme is less active, thereby resulting in the production of less infectious virions upon infection of a host animal. As the N, P and L proteins are involved in the RV polymerase activity, RV mutants having an insertion or deletion in the ORF encoding the N, P or L proteins are also part of the invention.

RV deletion and/or insertion mutants according to the invention can also be used to vaccinate a host in order to be able to discriminate (serologically) between a host to which a vaccine comprising said RV mutant is administered and a host infected with a parental RV. In this embodiment of the invention the insert in the RV insertion mutant may encode a heterologous epitope which is capable of eliciting a specific non-RV immune response in an inoculated host, or may encode a protein with enzymatic activity, such as CAT or lacZ (Conzelmann and Schnell, 1994, supra). A preferred region for the incorporation of such inserts is the RV pseudogene region. As is demonstrated in the Examples insertions and deletions can be made in this region without disrupting essential functions of RV such as those necessary for infection or replication. The RV deletion mutant may lack an epitope of a RV protein against which an immune response is normally raised by the vaccinates, in particular a RV mutant comprising a deletion in the ORF encoding the G protein is suited for this purpose. In the case of a RV insertion mutant the insertion comprises a nucleic acid sequence encoding a serological marker antigen or an epitope thereof.

In a further embodiment of the invention a RV mutant is provided which is capable of expressing one or more different heterologous epitopes or poly-peptides of a specific pathogen. Such a mutant can be used to vaccinate animals, both domestic and non-domestic animals, against wildlife rabies and said pathogen.

Vaccination with such a live vector vaccine is preferably followed by replication of the RV mutant within the inoculated host, expressing in vivo the heterologous epitope or polypeptide along with the RV polypeptides. The polypeptides expressed in the inoculated host will then elicit an immune response against both RV and the specific pathogen. If the heterologous polypeptide derived from the specific pathogen can stimulate a protective immune response, then the animal inoculated with the RV mutant according to the invention will be immune to subsequent infection by that pathogen as well as to infection by RV. Thus, a heterologous nucleic acid sequence incorporated into a suitable region of the RV genome may be continuously expressed in vivo, providing a solid, safe and longlasting immunity to the pathogen.

In particular, the present invention provides a RV vector which comprises an insertion of a nucleic acid sequence encoding an epitope or polypeptide of a specific pathogen, wherein the insertion is made in the pseudogene region.

If desired, part or whole of the pseudogene region can be deleted in the RV vector described above.

Preferably nucleic acid sequences encoding an epitope or polypeptide of canine parvovirus, canine coronavirus and classical swine fever virus (CSFV) are contemplated for incorporation into a suitable region of the RV genome.

The possibility to manipulate the non-segmented negative-stranded RNA genome of RV on the DNA level by recombinant DNA techniques was not possible until now, because no infectious replicating virus could be generated. However, a process is provided herein which allows the engineering of a mutation into a coding region or non-coding region of the viral genome on the DNA level by means of recombinant DNA techniques followed by the generating of an infectious replicating RV harbouring the mutation in its genome.

This process according to the invention comprises the steps of

a) introducing into cells expressing a RNA polymerase;

1) one or more DNA molecules encoding the RV N, P and L proteins, and
2) a DNA molecule comprising the RV cDNA genome

and
b) isolating the viruses produced by the cells.

Normally, the cDNA of the rabies virus genome is modified by the incorporation of a mutation in the genome.

The process may however also be used to e.g. purify contaminated RV pools. In that case, the original non-mutated cDNA will be used.

In view of the fact that rescue efficiency of a model mini-genome of RV comprising heterologous inserts with plasmid encoding proteins is extremely low and moreover correlates with insert length (Conzelmann and Schnell, 1994, supra) it could not be expected that initiation of a productive infection from transfected full length genomic RNA could be achieved by co-transfection with plasmids encoding the RV N, P and L proteins. This is the more so as large amounts of positive sense N, P and L specific RNAs are produced from the transfected protein encoding plasmids which were expected to hybridize with simultaneously expressed negative-stranded genomic RNA transcripts. Possible hybridization, however, which could affect more than half of the genome was suspected to interfere with the crucial encapsidation step. In addition, translation of N, P and L mRNA might be affected. Indeed it was found that with the standard transfection protocol no infectious viruses could be obtained. However, as demonstrated in the examples the application of an alternative transfection protocol in combination with the use of a RV cDNA genome generating positive stranded antigenomic RNA transcripts, gave rise to a replicating genetically engineered RV.

The above-mentioned process allows the in vitro incorporation of a mutation in the genome of a parental RV by means of recombinant DNA techniques followed by the generation of an infectious replicating RV mutant harbouring said mutation. The mutation includes but is not limited to an insertion, deletion or substitution of nucleic acid residues into an ORF encoding a RV protein, a non-coding region e.g. the pseudogene region, or a transcriptional signal sequence of RV parental genome.

The engineering of a mutation in a non-coding intergenic region may influence the transcription of a specific viral gene such that the transcription of the mRNA and the subsequent translation of the protein, either an envelope protein, such as the M and G protein or a protein involved in polymerase activity, such as the N, P or L protein, is reduced resulting in a virus mutant featuring attenuated characteristics because the mutant's capability of producing (infectious) progeny virus is reduced. In particular the substitution of one or more nucleic acid residues in this intergenic region and/or transcriptional signal sequences can influence efficiency of transcription.

Furthermore, the substitution of one or more nucleic acid residues in a region of the genome of a virulent RV which is involved with virulence, such as the ORF encoding the G protein, by the application of the process described herein is part of the invention.

Such a mutation may result in the exchange of a single amino acid in the G protein of a virulent RV strain resulting in a (partial) loss of pathogenicity, e.g. replacement of Arg (333) with Ile, Glu or Gln, or Leu (132) by Phe, or Trp.

In the process according to the invention the DNA molecule containing the RV genetic information preferably comprises a plasmid provided with appropriate transcription initiator and terminator sequences recognizable by a polymerase co-expressed by the transfected host cells.

A preferred process according to the invention comprises the use of host cells transfected with RV DNA, said cells being able to express bacteriophage T7 DNA-dependent RNA polymerase, expressed for example cytoplasmically from vaccinia virus recombinant. In this case the plasmids containing RV DNA are provided with the T7 promoter and terminator sequences (Conzelmann and Schnell, 1994, supra).

For the preparation of a live vaccine the recombinant RV mutant according to the present invention can be grown on a cell culture derived for example from BHK, or human diploid cells. The viruses thus grown can be harvested by collecting the tissue cell culture fluids and/or cells. The live vaccine may be prepared in the form of a suspension or may be lyophilized.

In addition to an immunogenically effective amount of the recombinant RV the vaccine may contain a pharmaceutically acceptable carrier or diluent.

Examples of pharmaceutically acceptable carriers or diluents useful in the present invention include stabilizers such as SPGA, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer).

Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminium hydroxide, phosphate or oxide, oil-emulsions (e.g. of Bayol F(R) or Marcol 52(R), saponins or vitamin-E solubilisate.

The useful dosage to be administered will vary depending on the type of mammal to be vaccinated, the age, weight and mode of administration.

The dosage may vary between wide ranges: 10^2 to 10^7 pfu/animal would e.g. be suitable doses.

A specific dosage can be for example about 10^6 pfu/animal.

A RV mutant according to the invention can also be used to prepare an inactivated vaccine.

For administration to animals, the RV mutant according to the present invention can be given inter alia orally, intranasally, intradermally, subcutaneously or intramuscularly.

The RV vaccine according to the invention can be administered to dogs but also to the main vectors, i.e. raccoons, skunks and foxes. Furthermore, also vaccination of wild boars with a live RV vector capable of expressing a heterologous gene of a porcine pathogen such as classical swine fever virus, is contemplated.

Example 1

Preparation of infectious replicating RV virions

Construction of full length RV cDNA (Fig. 2).

The cloning of cDNA spanning the entire genome of RV strain SAD B19 was described previously (Conzelmann et al., 1990, supra; GenBank accession number M31046). The numbering of RV nucleotides and amino acids used herein corresponds to that of Conzelmann et al., 1990 (supra). As basis for the assembly of a SAD B19 full length DNA clone the RV mini-genome sequence contained in the transcription plasmid pSDI-1 (Conzelmann and Schnell, 1994, supra) was used (Fig.2). pSDI-1 contains the SAD B19 genomic 3' and 5' ends (SAD B19 nucleotides 1-68 and 11760-11928, respectively) inserted between a T7 RNA polymerase promoter and the hepatitis delta virus (HDV) antigenome ribozyme sequence. In order to generate a plasmid to produce positive stranded SDI-1 transcripts (pSDI-1plus) the RV sequences contained in pSDI-1 were first amplified by PCR using an 11 base primer (5'-ACGCTTAACAA-3') which due to the complementary of RV genome ends corresponds to the 5' termini of both positive and negative sense viral RNAs. After subsequent partial ligation of a synthetic EcoRI/blunt adaptor (T7/3) containing a T7 promoter sequence followed by three G residues (underlined) (5'-AATTCCTGCAGTAATACGACTCACTATAGGG-3') to the amplified RV sequence, the ligation products were cloned in the EcoRI/SmaI sites of pX8dT. This plasmid is a derivative of pBluescriptII (Stratagene) from which a BssHII/ClaI fragment of the multiple cloning site containing the original T7 promoter was deleted. It contains the 84 base HDV antigenomic ribozyme sequence in the SmaI site followed immediately by a T7 transcription terminator sequence cloned in the BamHI site. Constructs that contained a T7 promotor upstream of the plus sense RV sequence were identified by restriction analysis and sequencing. The MunI-Bg1II fragment of pSDI-1 (SAD B19 nucleotides 40-68) was then replaced with a 1 kb MunI/Bg1II cDNA construct assembled in pBluescriptII from three fragments of different SAD B19 cDNA clones (MunI-SphI (SAD B19 nucleotides 40-482 from pZAD1-9); SphI-AatII (4041-4273 from pSAD13), and AatII-Bg1II (11472-11759 from pSAD85)) resulting in pSDI-1170. By insertion of a SphI fragment assembled from the clones pSAD25 and pSAD13 via NcoI (SAD B19 nucleotides 482-4041) and an AatII fragment assembled from clones pSAD 49 and pSAD85 via XhoI (SAD B19 nucleotides 4273-11472) into the unique SphI and AatII sites of pSDI-1170, the final basic full length clone pSAD L16 was completed. Using the circular plasmid, in vitro transcriptions were performed and the products analyzed on denaturing agarose gels. The presence of RNA transcripts comigrating with 12 kb RV genomic RNA indicated that full length antigenome RNA is transcribed by T7 polymerase.

Recovery of infectious recombinant RV

The co-transfection of plasmid pSAD L16 and plasmids encoding RV proteins N, P and L was carried out as described in Conzelmann and Schnell, 1994 (supra).

Transfection experiments were carried out as described previously. BHK-21, clone BSR cells were grown overnight in 3.2 cm-diameter dishes in Eagle's medium supplemented with 10% calf serum to 80% confluence, and infected at a m.o.i. of 5 with the recombinant vaccinia virus vTF7-3 (Fours et al., Proc. Natl. Acad. Sci USA 83, 8122-8126, 1986). One hour pontificating cells were washed twice with culture medium lacking calf serum and transfected with a plasmid mixture containing 5 µg pT7T-N, 2.5 µg pT7T-P, and 2.5 µg pT7T-L and with 2 µg of pSAD-L16 plasmid by using the mammalian transfection kit (Stratagene; $CaPO_4$ protocol) according to the suppliers instructions. The precipitate was removed 4 h posttransfection and cells were washed and incubated in Eagle's medium containing 10% calf serum. Possible encapsidation of pSAD-L16 derived T7 RNA polymerase transcripts and the resulting expression of RV proteins from the nucleocapsids was checked by indirect fluorescence. A monoclonal antibody directed against RV G protein, which could only be expressed from the recombinant RV genome, was used to screen the cultures. One day after transfection stained cells were present, demonstrating expression of genes from the RV genome. However, only single positive cells were observed in a series of 20 transfection experiments. No fluorescent cell foci indicating the presence of infectious virus were obtained in these experiments. In addition, from cell cultures which were inoculated with the entire supernatant from the transfected cells no infectious virus could be recovered two days later. Therefore, in order to isolate a presumed very low number of infectious virus generated in transfected cells, the experimental procedure was modified. For isolation of transfectant viruses cells and supernatants were harvested 2 days post transfection. Cells were suspended in the supernatant by scratching with a rubber policeman. The suspension was submitted to three cycles of freezing and thawing (-70 °C/37 °C, 5 min each). Cellular debris and the excess of vaccinia virus which forms aggregates under these conditions was pelleted by 10 min of centrifugation at

10.000 g in a microfuge. The entire supernatant was used to inoculate a culture dish with a confluent monolayer of cells. After incubation for 2 h, the supernatant was replaced by 2 ml of fresh culture medium. A cythopathogenic effect (cpe) caused by vaccinia virus was observed one to two days post infection. In average only ten plaques were observed after centrifugation at 10.000 g. RV infection of cells, which does not result in detectable cpe was demonstrated two days post infection by direct immunofluorescence staining of the entire monolayer with an anti-N conjugate (Centocore). In two out of 20 experiments fluorescent foci were observed and the respective supernatants contained infectious RV (SAD L16) which was assumed to represent transfectant virus generated from cDNA transcripts.

Half of the supernatants from the cultures in which foci were observed, was used for the second passage after centrifugation at 10.000 g. For further passaging (2 days each) decreasing aliquots of supernatants were used according to the degree of RV infection. To get completely rid of Vaccinia virus, supernatants from cultures approaching infection of all cells (third passage) were centrifuged two times for 10 min at 14.000 g in a microfuge. The final supernatant was then filtered using a sterile MILLEX-VV 0.1 µm filter unit (Millipore Products, Bedford, MA 01730) and then used to produce high titre stocks of recombinant RVs.

The latter transfection and isolation protocol was used in the subsequent Examples.

Example 2

Insertion of an oligonucleotide in the RV pseudogene region

Manipulations of the ψ were carried out in the subclone pPsiX8, containing a 2.8 kb XhoI-ScaI fragment of pSAD L16 representing SAD B19 nucleotides 3823 to 6668. The StuI fragments of the modified pPsiX8 plasmids were then isolated and used to replace the corresponding fragment (SAD B19 position 4014 to 6364) of the full length clone pSAD L16 (Fig. 1). Insertion of 4 nucleotides into the ψ and generation of a novel NheI site was achieved by digestion of pPsiX8 with Hind III, fill in of the extensions with Klenow enzyme and religation. The final full length clone pSAD U2 is distinguished from SAD L16 by the duplication of nucleotides 5338 to 5341.

The generation of infectious viruses was demonstrated after transfer of extracts from transfected cells together with supernatant to fresh cells. In each of the series focus formation was observed in one experiment. The transfectant viruses (clones SAD U2-13 and SAD U2-32) were passaged by transfer of supernatants to fresh cells two further times resulting in almost 100% infection of the cells. To demonstrate the insertion in the SAD U2 virus genome, total RNA was isolated from cells infected with SAD U2-13 and reverse transcriptase-PCR (RT-PCR) of the ψ was performed. With the primers G3P and L4M (Fig. 1), which are specific for the G and L genes, respectively, DNA fragments of approximately 730 bp were obtained from the genomes of transfectant viruses SAD U2 and SAD L16 and of standard RV SAD B19. However, subsequent digestion with HindIII was only observed for the PCR DNA obtained from SAD B19 and SAD L16, but not for that from SAD U2. Conversely, only SAD U2 derived DNA was digested with NheI, giving rise to two fragments of approximately 530 and 200 bp, respectively (Fig. 3). Direct RT sequencing of genomic RNA of transfectant virus SAD U2 further confirmed the presence of the expected insertion of 4 residues at the predicted site, while the rest of the determined sequence corresponded to that of the original SAD B19 genome. Thus, it was clear that SAD U2 virus represented a transfectant virus whose genome originated from engineered cDNA.

The introduction of four additional nucleotides close to the end of the RV ψ did not affect viability of the transfectant virus SAD U2, nor did it interfere with correct transcription termination of the G mRNA.

Example 3

Alteration of RV transcription by an insertion or deletion between G and L coding region

By double digest with StyI and HindIII, Klenow fill in and religation, 396 bases (SAD B19 nucleotides 4942 to 5337) were deleted, the final construct was pSAD W9. For the construction of pSAD V*, a 180 bp BglII-AsuII fragment including the SAD B19 N/P cistron border region was isolated from pSAD13 (Conzelmann et al., 1990, supra). The fragment contained 97 nucleotides of the N coding region, the entire 3' non-coding region and the N/P cistron border consisting of the N transcriptional stop/polyadenylation signal, the intergenic region, and the first 16 nucleotides of the P cistron including the transcriptional start signal. The cDNA fragment was first subcloned into the EcoRI site of pBluescript after fill-in of 3' recessive ends with Klenow enzyme (pNigP-180). After excision with HindIII/XbaI from pNigP and blunt end generation the obtained 230 bp fragment which contained the RV insert flanked by 16 and 34 bp of vector derived sequences, respectively, was cloned into the filled-in StyI of pPsiX8. The final full length construct (pSAD V*) thus possessed a 234 bp insertion compared to pSAD L16.

As before, pSAD V* and pSAD W9 were used to transfect twenty culture dishes each. In three cultures transfected with SAD V* and in one with SAD W9, rescue was indicated by subsequent isolation of viable virus. After five successive passages RNA from infected cells and supernatant was isolated and analyzed by RT-PCR using the same primers as in the previous experiments. In comparison to standard SAD B19 virus, an enlarged DNA fragment of approximately 0.9 kb resulted from RNA of cells infected with SAD V* thus showing that additional sequences were present in the ψ region of this transfectant virus (Fig. 4). In contrast, from RNA of cells infected with SAD W9, a DNA fragment of only 0.3 kb

was obtained; this size was expected according to the deletion made in the cDNA genome copy. Sequencing of PCR products confirmed further that the original engineered cDNA sequences were rescued into the genomes of SAD V* and SAD W9 transfectant viruses. Accordingly, neither the presence of additional sequences, including 50 vector derived nucleotides, between the G open reading frame and the ψ nor the deletion of the entire ψ did interfere with the infectivity and propagation of transfectant rabies viruses. The alterations engineered into the genomes of SAD V* and SAD W9 were designed in a way to result in phenotypical changes in the transcription pattern and it was investigated whether this affected the growth characteristics of the respective transfectant viruses. However, propagation in cell culture as well as final titers of infectious SAD V* and SAD W9 viruses were similar to those of standard SAD B19 RV. Three days after infection of cells with an m.o.i. of 0.01, titers of 10^8 focus forming units (ffu) were reached in the supernatants for SAD B19, SAD V* and SAD W9 demonstrating that the RV ψ is not essential for propagation in cell culture.

Using a ψ specific probe, no hybridization was detected with RNA from cells infected with the ψ-deleted SAD W9 virus. While the genomic RNAs of the other viruses and the G mRNAs of SAD B19 and SAD L16 were recognized by this probe, the SAD V* G mRNA did not react. In contrast, a faint band of RNA appeared that corresponded in size to the novel extra ψ-mRNA that was predicted by the presence of the extra P gene transcriptional start signal preceding the SAD V* ψ sequences. In contrast to naturally occurring RV, the transfectant virus SAD V* represents a RV whose genome is composed of six functional cistrons.

Example 4

Expression of a foreign protein-encoding gene from recombinant RV

The 230 bp cDNA fragment containing the N/P cistron border flanked by multiple restriction sites described in example 3 was introduced into the BstXI site of the pseudogene region of the full length cDNA pSAD L16 (SAD B19 position 4995) after generation of blunt ends with Klenow enzyme. The resulting cDNA pSAD V was used as a basis for introduction of the bacterial chloramphenicol-acetyltransferase (CAT) gene. To obtain pSAD XCAT, a 0.8 kb DNA fragment of pCM7 (Pharmacia) containing the entire CAT coding region was cloned into the AsuII site of pSAD V contained in the N/P cistron border upstream of the pseudogene sequence. For construction of pSAD VCAT, the cDNA between the AsuII site and the HindIII site located close to the end of the pseudogene sequence (SAD B19 position 5337) was deleted and replaced with the CAT-encoding HindIII-DNA from pCM7 after blunt end generation with Klenow enzyme. Accordingly, transcription of the recombinant RV SAD XCAT should give rise to a CAT mRNA possessing the pseudogene sequence as a nontranslated 3' region, whereas SAD VCAT should transcribe a CAT mRNA lacking the pseudogene sequence.

Recombinant rabies viruses were rescued after transfection of plasmids encoding RV N, P, and L proteins and pSAD-XCAT, and pSAD-VCAT, respectively, as described in Example 1. After removal of vaccinia virus, the transcription pattern of the recombinant RVs were analysed by northern hybridization. Both viruses transcribed CAT mRNAs of the expected size and composition (Fig. 5). The expression of CAT enzyme activity was determined in cells infected with the two viruses, respectively, by standard CAT assays (Conzelmann and Schnell, 1994, supra). Both were found to express CAT efficiently. Successive passages in cell culture cells showed that the introduced foreign sequences are genetically stable. Even after 40 passages both viruses expressed CAT efficiently (Fig. 6). Additional experiments were performed in order to examine expression and behaviour of the recombinant viruses in infected animals. Six week old mice (five each) were injected intracerebrally with 10^4 ffu of SAD VCAT, SAD XCAT, and standard sequence RV SAD L16, respectively. Seven days after infection all animals showed typical rabies symptoms and died from rabies within the following week. CAT activity was demonstrated in brains of mice infected with SAD VCAT and SAD XCAT, respectively. Both viruses could be reisolated from mouse brains and expressed CAT cell culture. Thus, a foreign gene can be introduced into the genome of infectious RV and be expressed stably and as well may serve as a marker to differentiate recombinant viruses.

Example 5

Expression of a heterologous viral antigen from recombinant RV and induction of an immune response against RV and the heterologous virus

The genome of classical swine fever virus (CSFV) encodes three structural glycoproteins (E0, E1 and E2). In CSFV infected animals neutralizing antibodies are directed against E2, whereas E0 induces a cellular immune response, cDNA encompassing the coding region of the E2 protein and the E0 protein of CSFV strain Alfort respectively. were used to replace the pseudogene region between the AsuII and HindIII sites of pSAD V as described in Example 4. Recombinant viruses (SAD-VE0 and SAD-VE2, respectively) were recovered from transfection experiments as detailed in Example 1. In infected cells the viruses expressed CSFV E0 protein, and CSFV E2 protein, respectively (Fig. 7).

The recombinant viruses SAD VE0 and SAD VE2 were used to immunize pigs by the oral route. Standard fox baits usually being used for oral immunization of foxes with the attenuated RV SAD B19 strain were loaded with 107 pfu of SAD-VE0, SAD-VE2 and SAD B19, respectively. Two baits of each preparation were fed to two pigs each (pig #1 and #2: SAD VE0, #3 and #4,

SAD B19, #5 and #6, SAD VE2). Four weeks after immunization, the presence of neutralizing antibodies against RV and CSFV as analysed. With the exception of #5, all pigs possessed RV neutralizing antibodies (titre >250) confirming uptake of the vaccine baits. Pig 5 was therefore not further considered. Pig #6 developed CSFV neutralizing antibodies at a titre of >16. As expected, pigs #1 to 4 did not develop CSFV neutralizing antibodies. An intranasal challenge with 10^7 pfu of CSFV strain Alfort was performed 5 weeks after immunization. Leucocyte numbers of pigs and body temperature were monitored after the challenge and shown in Figures 8 and 9, respectively. All pigs developed fever, but pigs #1 and #2 as well as #6 recovered more quickly. The control animal #4 died 15 days post challenge with typical CSFV symptoms, the control #3 was killed on day 21. The presence of CSFV neutralizing antibodies in the fig fed with SAD VE2 and the partial protection of the pigs that received either SAD VE0 or SAD VE2 demonstrate that both humoral and cellular immune resonses against two heterologous viruses may be induced by recombinant RV live vaccines after application by the oral route.

Example 6

Generation of an attenuated RV by introduction of a mutation into G gene sequences

In order to generate a virus propagating less efficiently than the standard virus SAD B19, a recombinant was prepared that possesses a mutated G protein.

For this purpose, the sequence encoding the last 46 amino acids of the G protein were deleted. First, the G protein coding plasmid, pT7T-G (Conzelmann and Schnell, 1994, supra) was digested with AflIII (position 4752 of the SAD B19 sequence) and EcoRV (the latter site is present in the multiple cloning site of the plasmid) and blunt ends were generated by Klenow enzyme. Ligation of the resulting AflIII and EcoRV ends resulted in the generation of a translation termination codon at the former AflIII sequence. A 0.3 kb DNA PpuMI-SMaI fragment containing the modified region was used to replace the authentic PpuMI-BstXI fragment 4469-4995 of pSAD L16. This manipulation resulted in the deletion of SAD B19 nucleotides 4753-4995 encoding the carboxyterminal 46 aa of the G protein cytoplasmic tail and part of the pseudogene sequence. A further result is the introduction of 18 vector-derived nucleotides immediately downstream of the new G translation termination codon.

Recombinant RV (SAD DCD) was recovered as described in Example 1. As expected, a truncated G protein was expressed in cells infected with SAD DCD (Fig. 10). Compared to standard sequence virus SAD L16, 100 fold lower titres were obtained with SAD DCD virus after infection of cells at an m.o.i. of 1. In addition, a reduced rate of spread in cell cultures was observed (Fig. 11), indicating that the truncation of the G protein resulted in reduced assembly of virions or reduced cell infectivity of virions. To analyse the behaviour of SAD DCD in infected animals, five mice were injected intracerebrally with 10^5 ffu of SAD DCD and 5 mice with the same dosis of SAD L16.

Example 7

Generation of a rabies virus G-minus (G^-) mutant by complementation in trans

In order to delete the entire G protein coding region from the RV genome, the full length clone pSAD UE (Example 2) was used. This clone differs from pSAD L16 by the presence of a unique NheI site within the non-translated 3' region of the G gene (SAD B19 position 5339). By partial digestion of pSAD U2 with PflMI (SAD position 3176) and complete digestion with NheI, subsequent fill-in by Klenow enzyme and religation, a cDNA fragment comprising SAD B19 nucleotides 3177-5339 was removed. The resulting clone pSAD dG was used in transfection experiments to recover recombinant virus. In addition to plasmids encoding N, P, and L proteins, however, a plasmid encoding the G protein was cotransfected with pSAD dG to complement the G deficiency of the viral genome. The resulting virus SAD dG was passaged to cells again transfected with the G encoding plasmid and infected with the vaccinia virus vTF-7-3 to provide G protein.

RNA transcripts of SAD dG were analyzed by Northern blotting experiments. After hybridization with an N specific probe, the SAD dG genome was found to be considerably smaller than the rabies virus wt genome reflecting the cDNA deletion of 2.1 kb. A probe spanning the entire G coding region, however, failed to hybridize with SAD dG RNAs demonstrating the lack of G encoding sequences (Fig. 12). The identity of the deletion was further confirmed by RT-PCR and sequencing.

Phenotypically complemented SAD dG was able to infect noncomplementing BSR cells, to replicate its genome and to express the genes encoded by the genome. However, it was not able to produce infectious virions and thus, infection could not spread to other cells (Fig. 13) or be transferred by passage of culture supernatants to other cell cultures.

Example 8

Complementation of G mutants by heterologous glycoproteins: directing virus to specific cells

To demonstrate that heterologous surface proteins may be incorporated functionally in the envelope of a recombinant virus, the G-mutant SAD dG was complemented by recombinant viral glycoproteins as described in Example 7 for the rabies virus G. Infectious pseudotype particles were generated that contained the spike proteins from Mokola virus, another member of the Lyssavirus genus, the rhabdovirus vesicular stomatitis virus (VSV; serotype New Jersey, genus vesiculovirus) and

from the retrovirus human immunodeficiency virus (HIV-1, strain NL-43).

Expression from transfected plasmids of the authentic Mokola and VSV-G protein and infection of cells with SADdG resulted in the formation of infectious pseudotype viruses. Compared to rabies virus G and the closely related Mokola virus G, however, a reduced titre was observed with VSV-G (10^4/ml in contrast to 10^6/ml). After replacement of the cytoplasmic and transmembrane domain sequence of VSV-G by the corresponding domains of the rabies virus G protein, however, 10^6 infectious particles were generated suggesting that the cytoplasmic domain of the RV G is directing the protein into the viral envelope.

The generation of pseudotype particles containing authentic HIV gp 160 (gp 120/40) spikes was not observed. In contrast, expression of a chimeric protein composed of the ecto-and transmembrane domain of the HIV gp fused to the cytoplasmic domain of RV G resulted in the formation of RV(HIV) pseudotypes. This confirmed that the cytoplasmic domain of the G protein is responsible for efficient incorporation of spike proteins into the envelope of rhabdoviruses. The RV(HIV) pseudotype particles successfully infected Vero cells expressing the human CD4 surface protein ($T4^+$ cells) but not the control cells expressing CD8 ($T8^+$ cells) (cells were obtained from the AIDS Research and Reference Reagent Programme). The pseudotype viruses thus possess the host range and cell specificity of HIV.

LEGENDS TO FIGURES

Fig. 1:

Organization of the RV pseudogene region (ψ) and construction of recombinant RV genomes (drawn to scale). Numbers indicate the nucleotide positions in the anti-genome sequence of SAD B19. On top, the entire RV genome with its five open reading frames is shown. Mutations were carried out in pPsiX8 containing part of the genome (3823-6668) and reintroduced into the full length clone pSAD L16 by exchange of the StuI fragment (4014-6364). In the detail drawing, coding regions are represented by gray boxes, non-coding sequences as lines. Functional transcriptional signal sequences are indicated by filled bar (stop/polyadenylation) and arrowhead (mRNA transcription start). The non-functional signal-like sequence defining the start of the ψ region is shown by the open bar. Arrows indicate the position of oligonucleotide primers G3P and L4M used for RT-PCR analysis of the ψ region. In SAD U2, fill-in of HindIII extensions resulted in insertion of 4 nucleotides and generation of a unique NheI-site. In SAD V*, a cDNA fragment containing the RV N/P cistron border (SAD B19 nucleotides 1323-1502) was inserted into the StyI site; SAD W9 possesses a deletion of the StyI/HindIII fragment.

Fig. 2:

Simplified scheme for the construction of transcription plasmids containing full length RV cDNA. Numbers refer to nucleotide positions of the SAD B19 RV antigenome sequence (Conzelmann et al., 1990). The plasmid pSDI-1plus which served as a basis for reconstruction of full length RV genomic DNA is a counterpart of pSDI-1 (Conzelmann and Schnell, 1994) containing the SDI-1 RV mini-genome that comprises the terminal nucleotides 1-68 and 11760-11928 in opposite direction with respect to T7 RNA polymerase promoter (T7) and hepatitis delta virus antigenomic ribozyme sequence (HDV). The MunI-Bg1II fragment of pSDI-1plus was replaced with a 1 kb cDNA construct that was assembled from three SAD B19 cDNA clones as indicated. Insertion of a 3.6 kb SphI and a 7.2 kb AatII fragment which were assembled from two cDNA clones each resulted in the final plasmid pSAD L16 containing full length SAD B19 cDNA. Transcription of this plasmid by T7 RNA polymerase should yield positive stranded (antigenomic) RNA possessing three extra non-viral G residues at the 5' and a precise 3' end after autolysis of the ribozyme. (T7) T7 promoter; (T7T) T7 transcription terminator; (HDV) HDV antigenomic ribozyme sequence.

Fig. 3:

Demonstration of the genetic tag in the genome of the transfectant virus SAD U2.

Total RNA from cells infected with standard RV SAD B19 (B19) and transfectant viruses SAD L16 (L16) and SAD U2 (U2) was isolated 2 days post infection and used for RT-PCR amplification of the respective ψ regions with primers G3P and L4M. The amplified DNA was separated in a 1% agarose gel directly and after digestion with HindIII and NheI, respectively. A NheI restriction site is present only in DNA derived from SAD U2.M,DNA size marker.

Fig. 4:

PCR analysis of SAD B19 (B19), SAD V* (V*), and SAD W9 (W9) genomes. RT-PCR was performed as described in fig. 3 with primers G3P and L4M. Amplification products were separated in a 1% agarose gel.

Fig. 5:

Demonstration of CAT mRNAs transcribed by recombinant RVS.

A Northern blot of total RNA from cells infected with SAD L16 (L16), SAD XCAT (X6) and SAD VCAT (VC18), was hybridized with probes specific for the G gene (G), pseudogene-(Y), and CAT gene, respectively. On the left side the viral genomes (v) and particular mRNAs are indicated. While SAD XCAT transcribes an mRNA containing both CAT and pseudogene sequences ("CATY"), SAD VCAT lacks pseudogene sequences and tran-

scribes an mRNA ("CAT") possessing only CAT sequences. The size of RNA markers are given in kb.

Fig. 6:

CAT activity of SAD XCAT and SAD VCAT after multiple passages in cell culture. Cells were infected with viruses from the particular passages (number of passage as indicated) and equal amounts of cell extracts were analysed for CAT activity two days post infection. In lane "-" extracts from cells infected with SAD L16 were analysed.

Fig. 7:

Expression of E0 and E2 protein by recombinant RVs.

Cells were infected with SAD VE0 (isolates 1, 2, 3) and SAD VE2 (isolates a, b, c), respectively. Two days post infection, cell extracts were separated in PAA Gels under reducing conditions and transferred to nitrocellulose membranes. After incubation with monoclonal antibodies directed against CSFV $E0^-$ and E2 protein, respectively, and subsequently with a secondary antibody coupled to alkaline phosphatase, the proteins were visualized by addition of substrate and exposure to an X-ray film. As a control, baculovirus expressed and purified E0 and E2 protein was used (B). In addition, extracts from cells infected with CSFV (V) served for comparison.

Fig. 8:

Leucocytes of pigs immunized with SAD VE0 (#1 and 2), SAD VE2 (#6) and standard rabies virus SAD B19 (#3 and #4), and challenged with CSVF. Leucocyte amounts are given in percent of absolute numbers present prior to challenge (day 0). * (#1, day 10 p.ch.): not done, estimated value.

Fig. 9:

Body temperature of pigs after CSFV challenge (day 0).

a. Animals immunized with SAD VE0 (#1 and #2) developed mild fever until day 11 (#1) or no fever (#2). Both control animals immunized with SAD B19 (#3 and #4) showed high fever over a long period. #4 died at day 15 post challenge from classical swine fever, due to heavy symptoms, #4 was killed 21 days post challenge.

b. The animal immunized with SAD VE2 developed mild fever only at days 6 to 8. Controls are the same as in a).

Fig. 10:

Expression of a truncated G protein in cells infected with SAD DCD.

BSR cells were infected at an moi of 1 with SAD DCD or SAD L16 and at 16 h post-infection labelled with 50 µCi of [^{35}S]methionine for 3 h. Cell extracts were incubated with an anti-rabies G MAb and aliquots of immunoprecipitated samples were either digested with PNGase F (+PF) in order to demonstrate the protein backbones or mock treated (-) to demonstrate the glycosylated proteins. +TM: infected cells were incubated in the presence of 2 µg/ml tunicamycin for 90 min prior to labelling and during the 3 h labelling period. Proteins were separated on 10% SDS-PAGE and visualized by autoradiography. Cell extracts were analysed as above. L16, SAD L16 virus; ΔCD, SAD DCD mutant virus. M: Protein size markers.

Fig. 11:

Spread of SAD L16 and SAD DCD in cell culture.
Culture cells were infected at an m.o.i. of 0.05 with SAD L16 (L16) and SAD DCD (DCD), respectively, and analysed at the indicated times post infection by direct immunofluorescence with a conjugate (Centocor®) directed against rabies virus N protein. A slower spread of infection of neighbouring cells is observed in cells infected with SAD DCD.

Fig. 12:

Analysis of SAD dG (Example 7) and SAD dCD (Example 6) specific RNA' s.
Total RNA of BSR cells infected with SAD L16 (Example 1), SAD dCD (ΔCD) and phenotypically complemented SAD dG virus (ΔG) at m.o.i.s. of 1 was isolated 2 days post infection and analyzed by Northern hybridization. As demonstrated by hybridization with an N gene specific probe (A), the genome of SAD dG is considerably smaller than the standard rabies virus genome (v), reflecting the 2.1 kb deletion of the G gene. A probe spanning the entire G protein encoding sequence fails to hybridize with SAD dG RNAs. The small deletion of the cytoplasmic domain encoding region in the SAD dCD genome is demonstrated by the appearance of a G mRNA (G) that is shorter than the standard rabies virus G mRNA.
v: genomic RNA; N, G: monocistronic mRNAs; N+P, M+G, G+L: bicistronic mRNAs

Fig. 13:

Lack of spread of the G^- mutant SAD dG.
BSR cells were infected with phenotypically complemented SAD dG and analyzed 36 hours post transfection by immunofluorescence microscopy. In (A) N protein expression is shown by incubation of cells with a FITC-coupled antibody directed against N protein (Centocore). Only single cells are infected, no spread of virus to neighbouring cells is observed. (B): control with a G specific antibody.

Fig. 14:

Composition of the functional chimeric HIV/RV glycoprotein used for generation of RV(HIV) pseudotype virions. The entire HIV-NL43 gp160 cytoplasmic domain except for three amino acids directly downstream of the transmembrane domain was replaced by the complete RV-G cytoplasmic domain. "p" represents a proline residue not present in the parental proteins. Cytoplasmic and transmembrane domain sequences are separated by a slash (/).

Claims

1. A genetically manipulated infectious replicating non-segmented negative-stranded RNA virus mutant comprising an insertion and/or deletion in an open reading frame, a pseudogene region or an intergenic region of the virus genome.

2. A virus mutant according to claim 1, characterized in that the virus mutant comprises an insertion and/or deletion in a pseudogene region.

3. A virus mutant according to claim 1, characterized in that the virus mutant comprises an insertion and/or deletion in an open reading frame.

4. A virus mutant according to claim 3, characterized in that the virus mutant comprises an insertion and/or deletion in the open reading frame encoding the Matrix protein or an analog thereof, resulting in the absence of a functional Matrix protein , said mutant being phenotypically complemented with the Matrix protein.

5. A virus mutant according to claim 3, characterized in that the virus mutant comprises an insertion and/or deletion in the open reading frame encoding the glycoprotein G.

6. A virus mutant according to claim 5, characterized in that the insertion and/or deletion results in the absence of a functional glycoprotein G, said mutant being phenotypically complemented with a glycoprotein G analog.

7. A virus mutant according to claim 6 characterised in that the glycoprotein G analog is the rabies glycoprotein G.

8. A virus mutant according to claims 1-7, characterised in that it carries a heterologous nucleic acid sequence encoding an epitope or polypeptide of a pathogenic virus or microorganism.

9. A virus mutant according to claims 1-8, characterised in that the virus mutant belongs to the family of paramyxoviridae.

10. A virus mutant according to claims 1-8, characterised in that the virus mutant belongs to the family of rhabdoviridae.

11. A virus mutant according to claim 10, characterised in that the virus mutant is rabies virus.

12. A vaccine for the prevention of infection caused by a non-segmented negative-stranded RNA virus in a mammal, characterized in that the vaccine comprises a virus mutant according to claims 1-11 and a pharmaceutically acceptable carrier or diluent.

13. A process for the preparation of an infectious replicating non-segmented negative-stranded RNA virus comprising the steps of:

    a) introducing into a host cell expressing a RNA polymerase;

        1) one or more DNA molecules encoding the virus N, P and L proteins, or analogs thereof;

    and

        2) a DNA molecule comprising the cDNA of the non-segmented negative-stranded RNA virus genome and

    b) isolating the viruses produced by the cells.

14. A process according to claim 13, characterised in that the cDNA of the non-segmented negative-stranded RNA virus genome is modified by the incorporation of a mutation.

15. A process according to claims 13-14, characterized in that the transcripts of the non-segmented negative-stranded RNA virus cDNA genome are positive stranded antigenomic RNAs.

16. A process according to claims 13-15, characterized in that the RNA polymerase is T7 RNA polymerase, preferably expressed from a recombinant vaccinia virus.

17. A process according to claims 13-16, characterized in that the non-segmented negative-stranded RNA virus genome is obtained from the family of paramyxo-viridae.

18. A process according to claims 13-16, characterized in that the non-segmented negative-stranded RNA virus genome is obtained from the family of rhabdoviridae.

19. A process according to claim 18, characterized in that the non-segmented negative-stranded RNA virus genome is obtained from rabies virus.

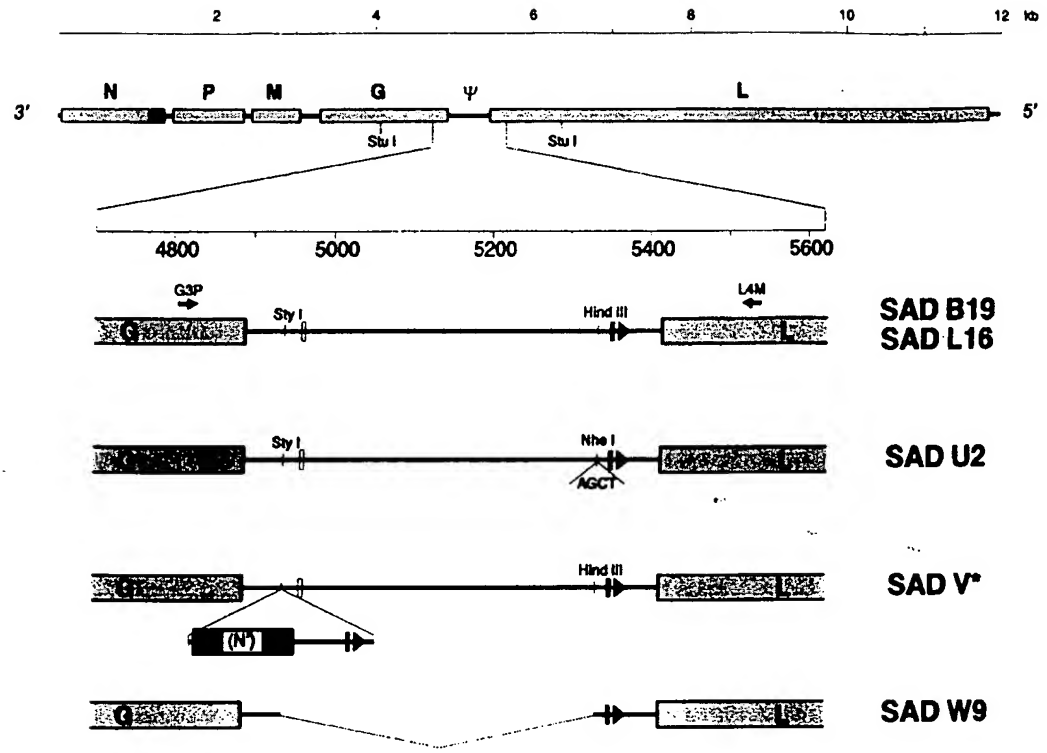

2
4
6
8
10
12 kb
3'
N
P
M
G
Ψ
L
5'
Stu I
Stu I
4800
5000
5200
5400
5600
G3P
Sty I
Hind III
L4M
SAD B19
SAD L16
Sty I
Nhe I
AGCT
SAD U2
Hind III
(N')
SAD V*
SAD W9

Figure 1.

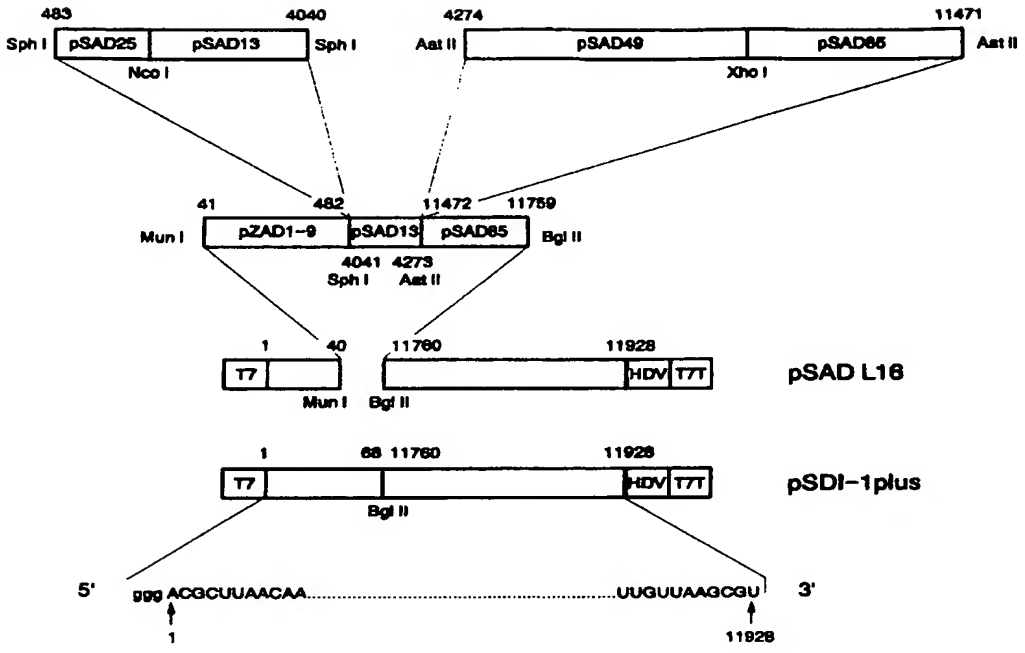

483
4040
4274
11471
Sph I
pSAD25
pSAD13
Sph I
Ast II
pSAD49
pSAD65
Ast II
Nco I
Xho I
41
482
11472
11759
Mun I
pZAD1-9
pSAD13
pSAD65
Bgl II
4041
4273
Sph I
Ast II
1
40
11760
11928
T7
HDV
T7T
pSAD L16
Mun I
Bgl II
1
68
11760
11928
T7
HDV
T7T
pSDI-1plus
Bgl II
5'
ggg ACGCUUAACAA.....UUGUUAAGCGU
3'
1
11928

Figure 2.

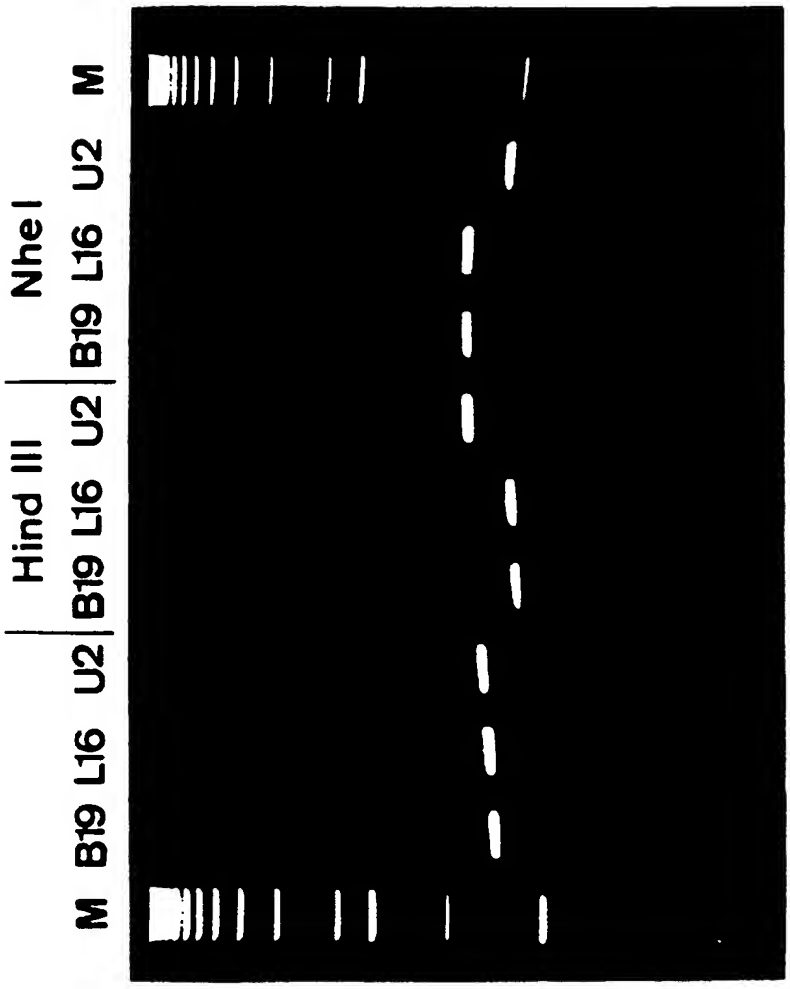


Figure 3.

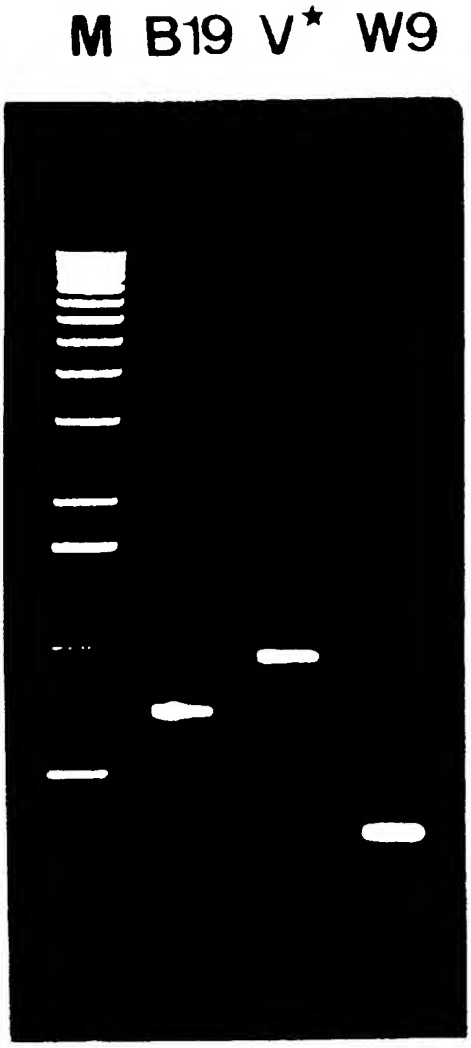


Figure 4.

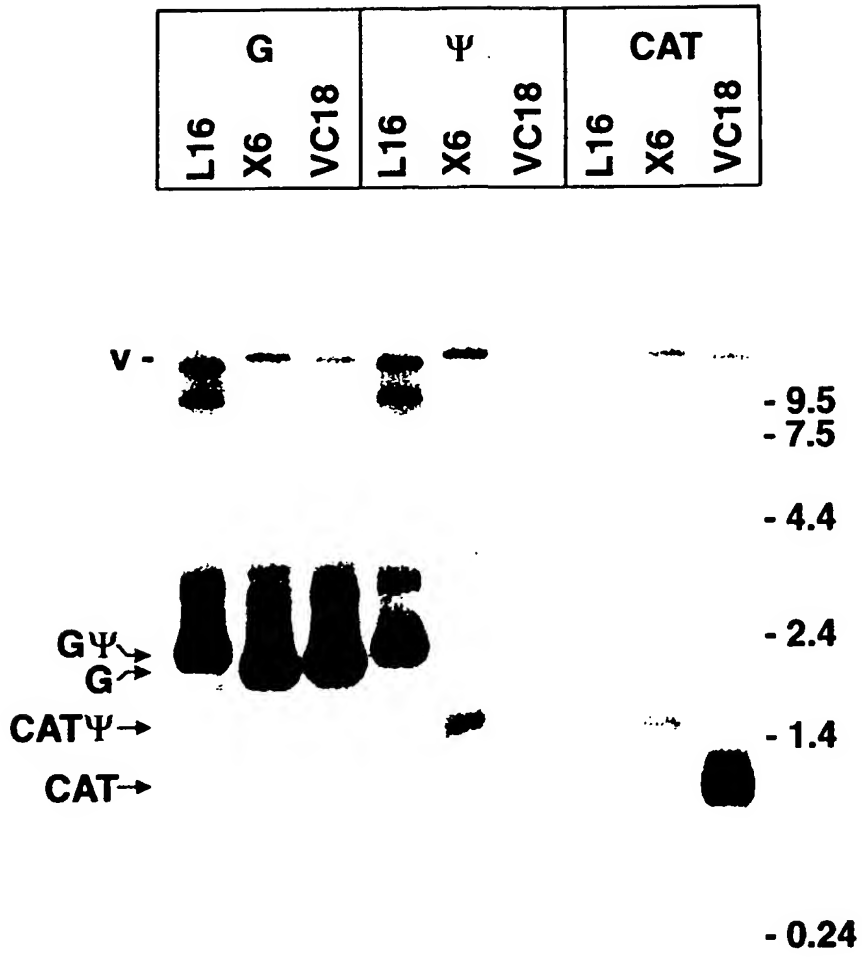

G
Ψ
CAT
L16
X6
VC18
L16
X6
VC18
L16
X6
VC18
V -
- 9.5
- 7.5
- 4.4
- 2.4
- 1.4
- 0.24
GΨ
G
CATΨ
CAT

Fig. 5

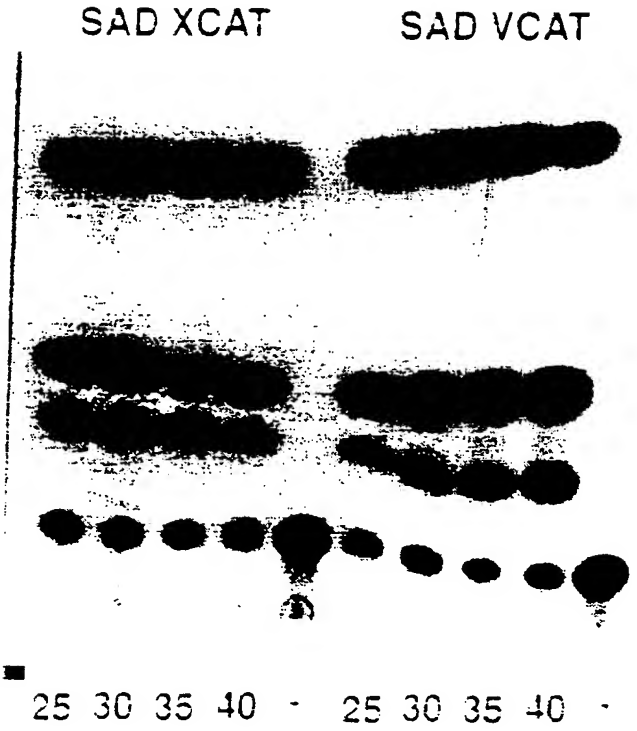
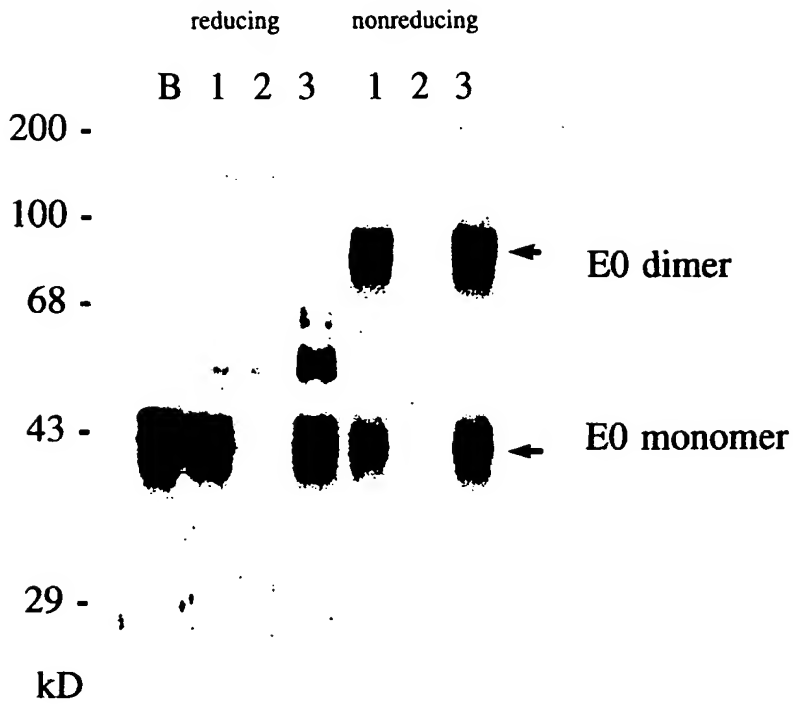


Fig. 6


reducing
nonreducing
B 1 2 3 1 2 3
200 -
100 -
68 -
43 -
29 -
kD
E0 dimer
E0 monomer

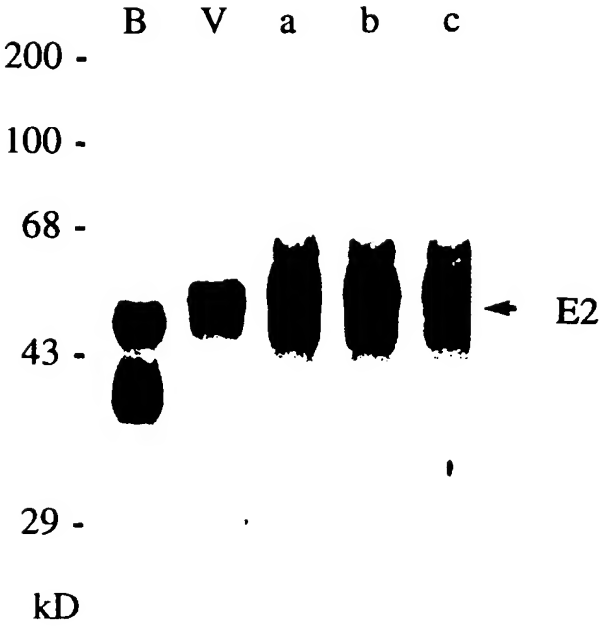

B V a b c
200 -
100 -
68 -
43 -
29 -
kD
E2

Fig. 7

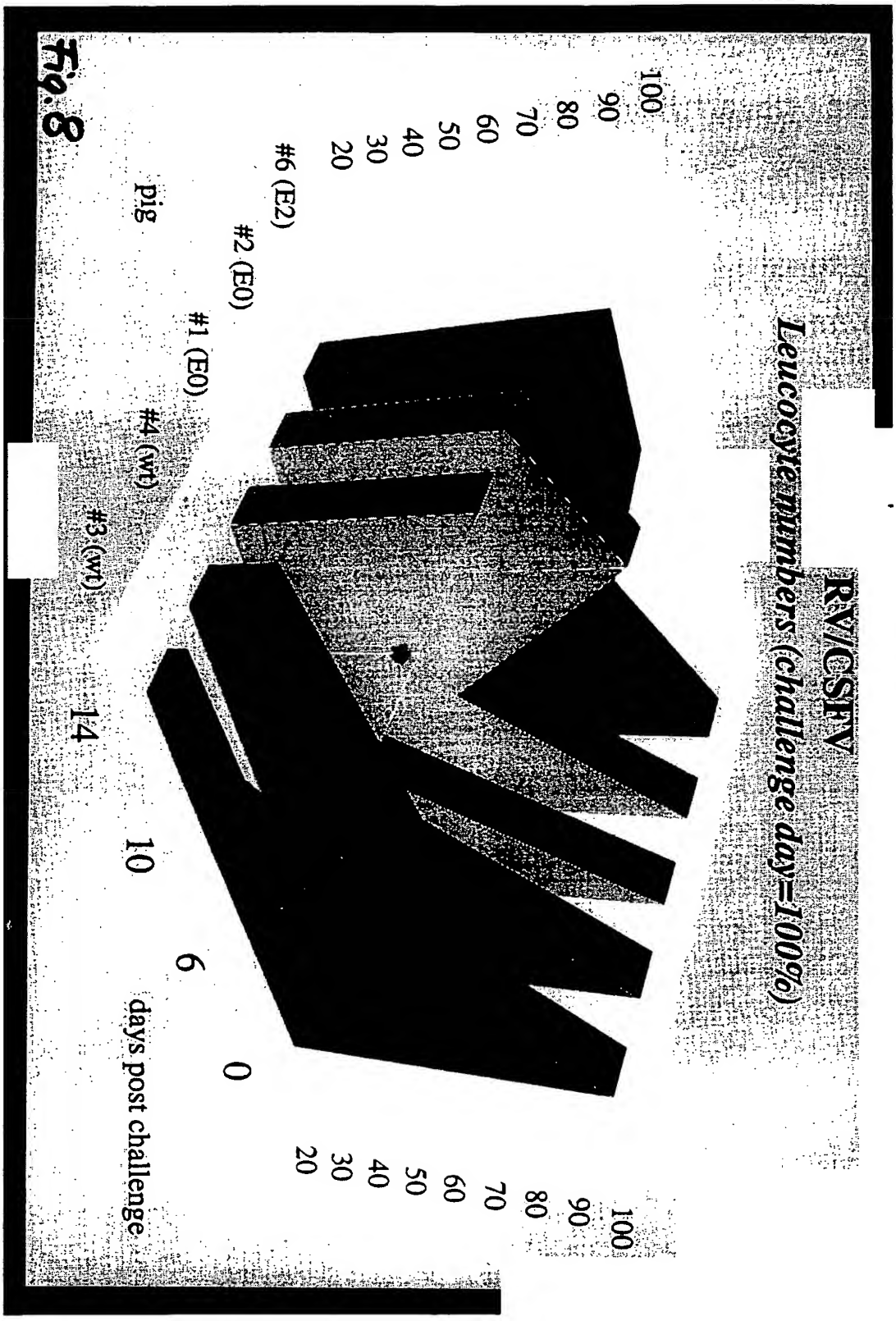

RV/CSFV
Leucocyte numbers (challenge day=100%)
100
90
80
70
60
50
40
30
20
#6 (E2)
#2 (E0)
#1 (E0)
#4 (wt)
#3 (wt)
pig
0
6
10
14
days post challenge

Fig. 8

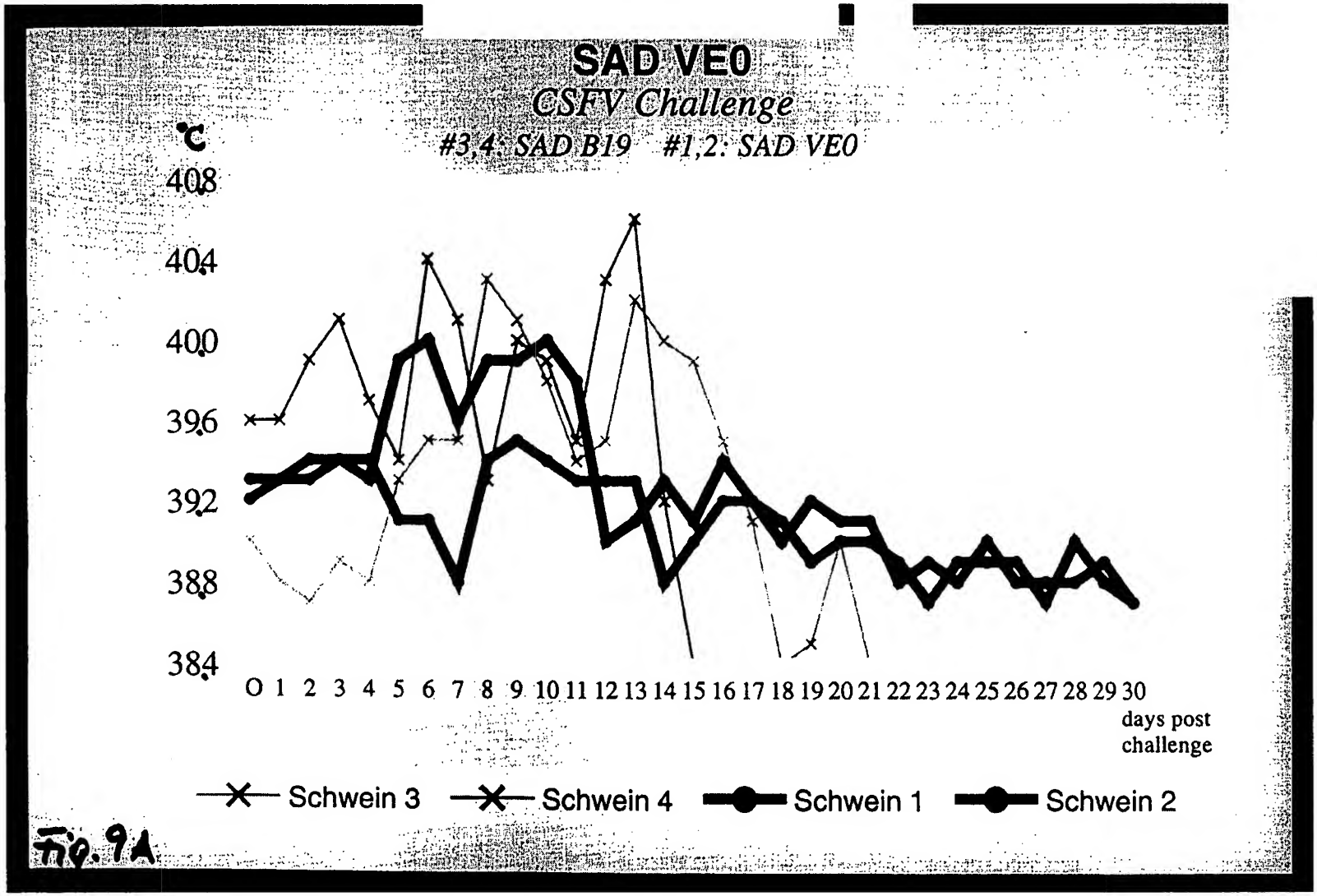

SAD VE0
CSFV Challenge
#3,4: SAD B19 #1,2: SAD VE0
°C
40.8
40.4
40.0
39.6
39.2
38.8
38.4
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
days post challenge
Schwein 3
Schwein 4
Schwein 1
Schwein 2

Fig. 9A

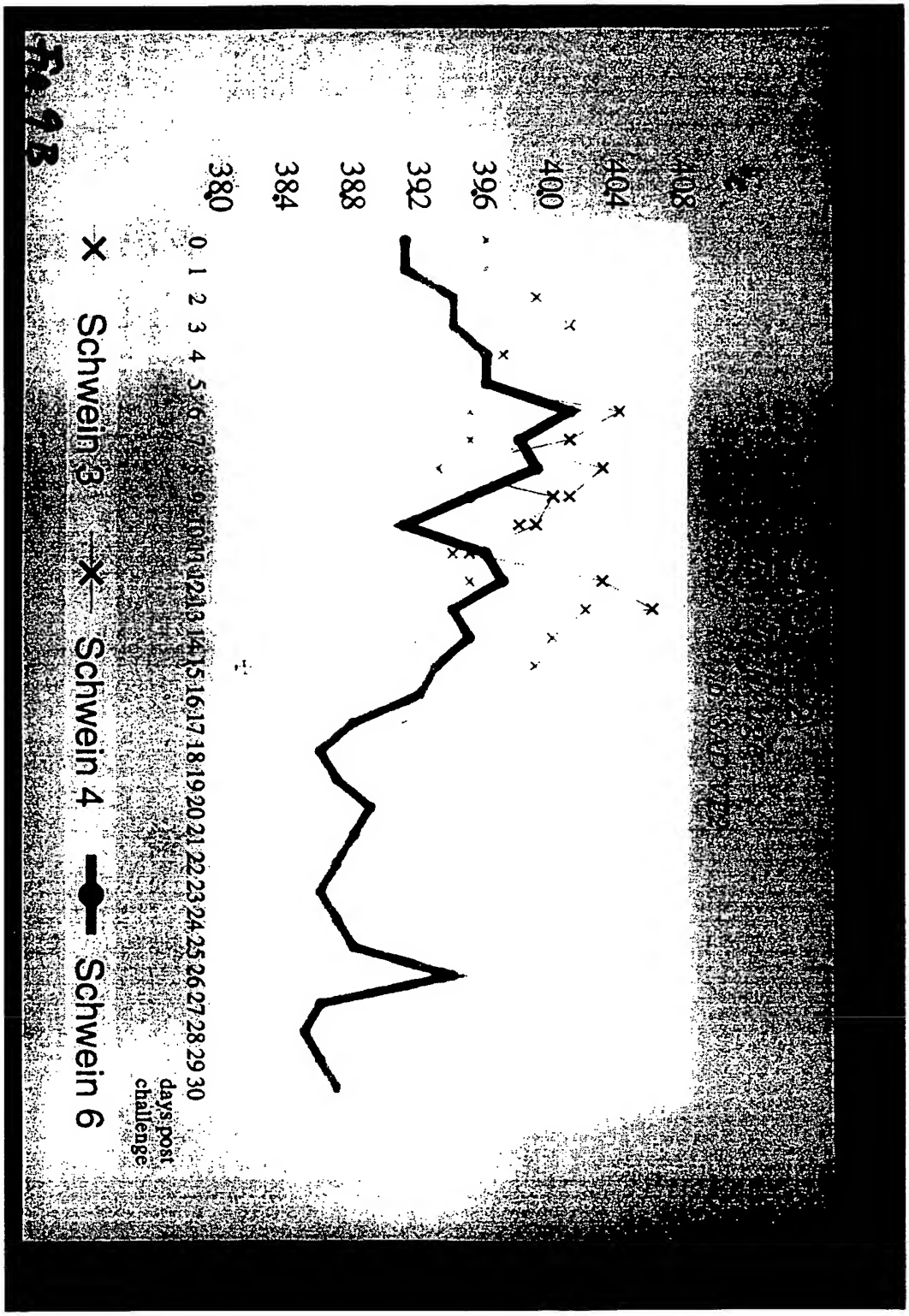
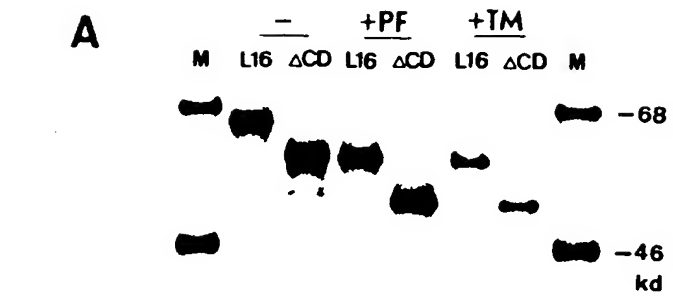

408
404
400
396
392
388
384
380
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
days post challenge
Schwein 3
Schwein 4
Schwein 6

Fig. 13


A
–
+PF
+TM
M
L16
ΔCD
L16
ΔCD
L16
ΔCD
M
-68
-46
kd

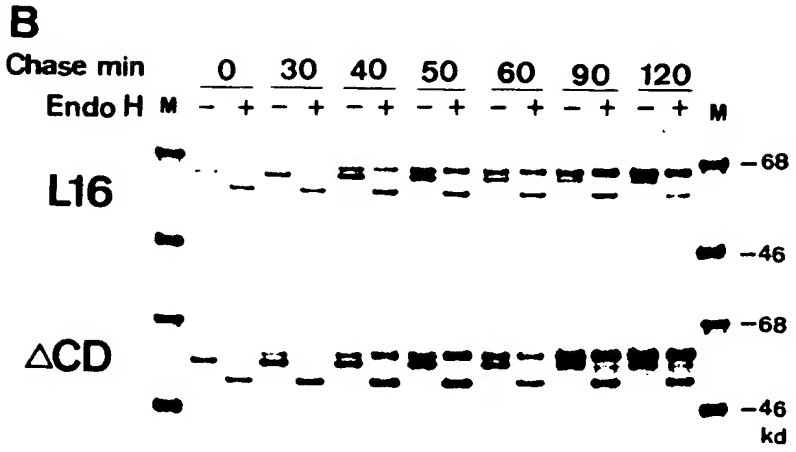

B
Chase min
0
30
40
50
60
90
120
Endo H
M
– + – + – + – + – + – + – + M
L16
ΔCD
-68
-46
-68
-46
kd

Fig. 10

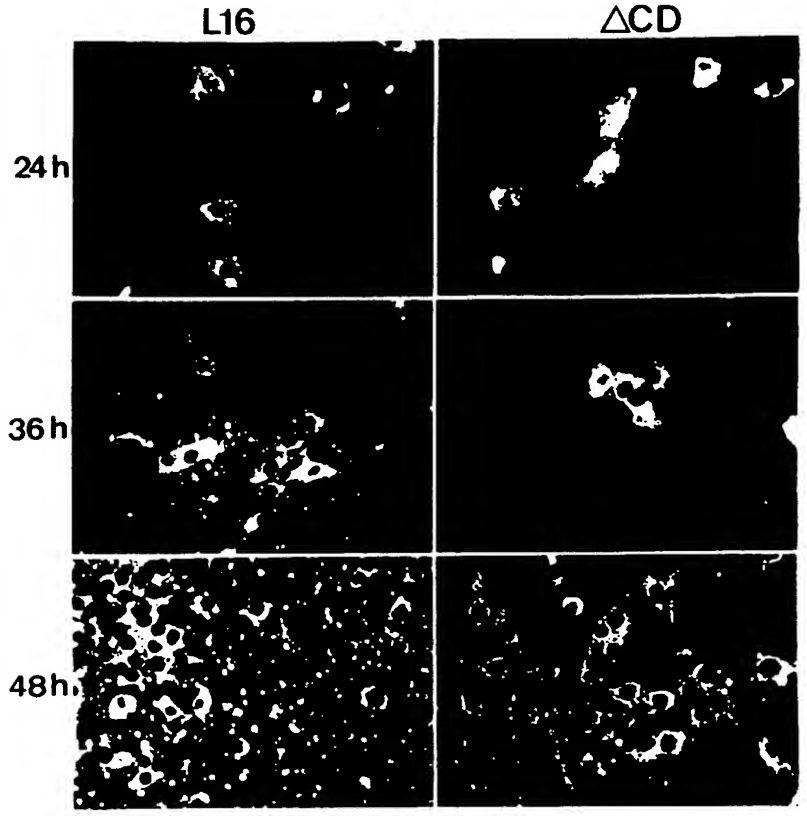


Fig 11

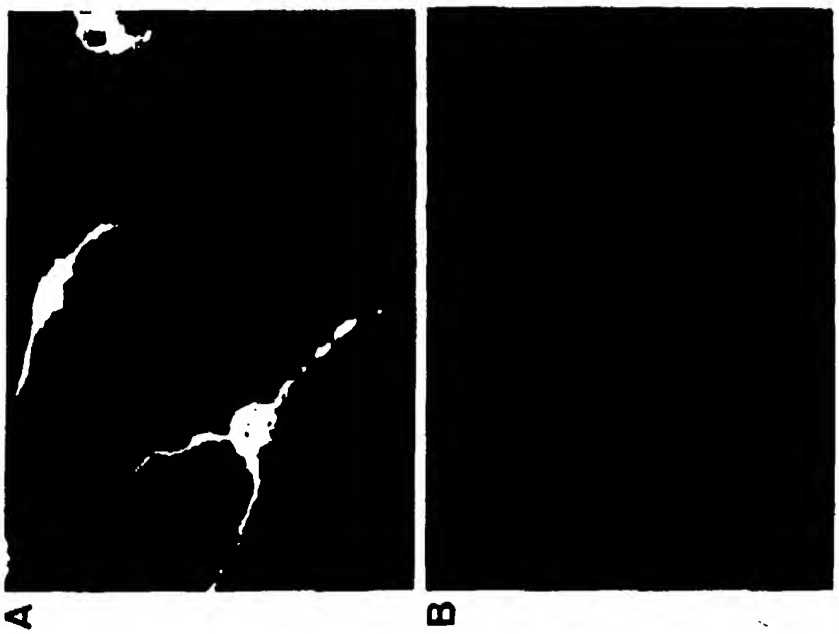


Fig. 13

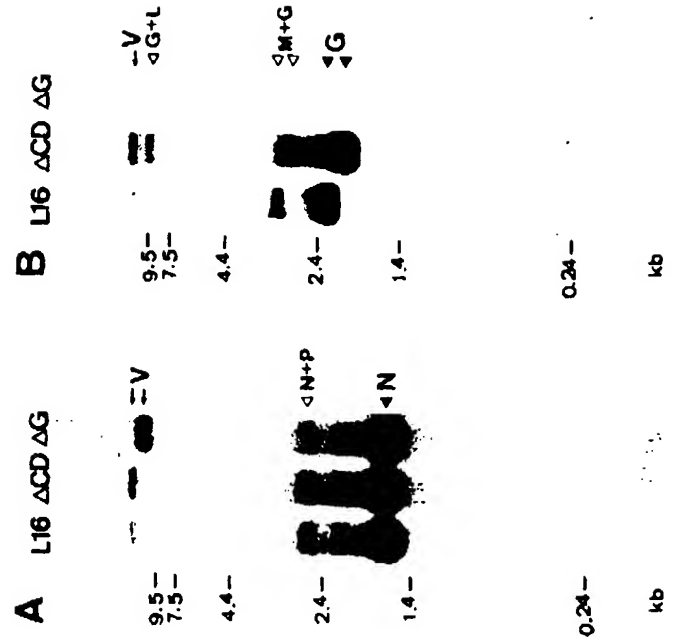
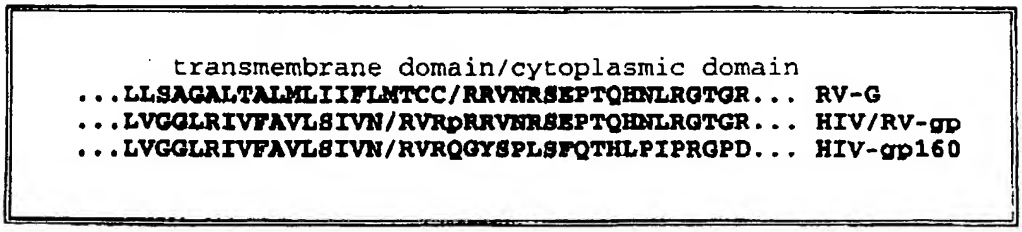


Fig. 12

Fig. 14


transmembrane domain/cytoplasmic domain
...LLSAGALTALMLIIFLMTCC/RRVNRSEPTQHNLRGTGR... RV-G
...LVGGLRIVFAVLSIVN/RVRpRRVNRSEPTQHNLRGTGR... HIV/RV-gp
...LVGGLRIVFAVLSIVN/RVRQGYSPLSFQTHLPIPRGPD... HIV-gp160

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 95 20 1936

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A,D	J. VIROL., vol. 68, no. 2, 1994 pages 713-719, K. K. CONZELMAN ET AL. 'Rescue of synthetic genome analogs of rabies virus by plasmid encoded proteins' * the whole document * ---	1	C12N15/86 C12N15/47 C07K14/145 A61K39/205 C07K14/16 C07K14/185
A,D	CELL, vol. 59, no. 6, 1989 pages 1107-1113, W. LUYTJES ET AL. 'Amplification, packaging and expression of a foreign gene by influenzy virus' * the whole document * ---	1	
A	J. VIROL., vol. 67, no. 12, 1993 pages 6659-6666, S. LI ET AL. 'Chimeric influenza virus induces neutralising antibodies' * the whole document * ---	1	
A	PROC. NATL. ACAD. SCI. USA, vol. 88, 1991 pages 1379-1383, A. PATTNAIK ET AL. 'Cells that express all five proteins from VSV from cloned cDNAs support replication, assembly and budding of defective interfering particles' * the whole document * ---	1	
	-/--		

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

C12N
C07K
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	2 January 1996	Skelly, J

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 95 20 1936

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	EMBO J., vol. 9, 1990 pages 379-384, I. BALLART ET AL. 'Infectious measles virus from cloned cDNA' * the whole document * ---	1	
A	EMBO J., vol. 10, no. 11, 1991 page 3358 D. ESCHLE ET AL. 'Retraction: Infectious measles virus from clined cDNA' ---	1	
A	EP-A-0 440 219 (SCHWEIZ SERUM & IMPFINSTITUT BERN) * the whole document * ---	1	
A	WO-A-94 08022 (COMMONWEALTH SCINTIFIC AND INDUSTRIAL REEARCH ORGANISATION) * the whole document * ---	1	
A	WO-A-91 03552 (MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK) * the whole document * ---	1	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
X,P	EMBO J., vol. 13, no. 18, 1994 pages 4195-4203, M. SCHNELL ET AL. 'Infectious rabies virus from cloned cDNA' * the whole document * -----	1-19	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	2 January 1996	Skelly, J

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)

HIGH POTENCY RECOMBINANT ANTIBODIES AND METHOD FOR PRODUCING THEM

FIELD OF THE INVENTION

The present invention relates to high potency antibodies as well as methods of increasing the potency of antibodies, as well as producing antibodies with initially high potency, and to methods of using such antibodies for purposes of prevention and treatment of diseases.

BACKGROUND OF THE INVENTION

Antibodies have been, and are currently being, developed for the prevention and treatment of various diseases, especially those caused by infectious microorganisms, most especially viruses.

One approach has been the development of antibodies, especially neutralizing monoclonal antibodies, with high specific neutralizing activity. One drawback to this route has been the need to produce human antibodies rather than those of mouse or rat and thus minimize the development of human anti-mouse or anti-rat antibody responses, which potentially results in further immune pathology.

One alternative approach has been the production of human-murine chimeric antibodies in which the genes encoding the mouse heavy and light chain variable regions have been coupled to the genes for human heavy and light chain constant regions to produce chimeric, or hybrid, antibodies. For example, a humanized anti-RSV antibody has been prepared and is currently being marketed. [See: Johnson, U.S. Pat. No. 5,824,307]

In some cases, mouse CDRs have been grafted onto human constant and framework regions with some of the mouse framework amino acids being substituted for correspondingly positioned human amino acids to provide a "humanized" antibody. [Queen, U.S. Pat. No. 5,693,761 and 5,693,762]. However, such antibodies contain intact mouse CDR regions and have met with mixed effectiveness, producing affinities often no higher than 10^7 to 10^8 M^{-1}.

The production of ultra high affinity antibodies, especially where such antibodies exhibit a high potency (i.e., biological activity, such as antigen neutralizing ability) would be desirable from the point of view of both the neutralizing ability of such an antibody as well as from the more practical aspects of needing to produce less antibody in order to achieve a desirable degree of clinical effectiveness, thereby cutting costs of the accompanying use.

Antibody affinity is measured by the binding constant of the antibody for a particular antigen, and such binding constant is calculated by the ratio of the rate constant for antibody-antigen complex formation (referred to as the "k_{on}" value) to the rate constant for dissociation of said complex (the "k_{off}" value). In accordance with the present invention, regardless of the value of the affinity constant, or association constant, of the antibody for the particular antigen, the potency, or biological activity, of the antibody is a

function of the value of k_{on}. Stated another way, applicants herein disclose a solution to problems of achieving high antibody potency (i.e., biological activity, such as virus neutralization) related to the observation that for the ratio of k_{on} to k_{off} (i.e., the binding affinity), the higher the k_{on} value, the higher the potency of the antibody. It is another object of the present invention to provide methods for producing antibodies with sufficiently high values of k_{on} so as to yield useful potency characteristics.

BRIEF SUMMARY OF THE INVENTION

In accordance with an aspect of the present invention, there are provided antibodies that may be used in the treatment and/or prevention of a disease wherein such antibodies have increased potency.

In accordance with an aspect of the invention, applicants have found that the potency of an antibody may be increased by increasing the rate constant for antigen-antibody complex formation, which is referred to as the "k_{on}" value.

In one aspect, the present invention relates to high potency neutralizing antibodies, including immunologically active portions, fragments, or segments thereof, having a k_{on} of at least 2.5×10^5 M^{-1} s^{-1}, preferably at least about 5×10^5 M^{-1} s^{-1}, and most preferably at least about 7.5×10^5 M^{-1} sec^{-1}.

In another aspect, the present invention is directed to methods of producing high affinity neutralizing antibodies with high specificity and potency, or biological activity, preferably having an affinity of at least about

10^9 M^{-1}, and having a k_{on} value of at least about 2.5 x 10^5 M^{-1} sec^{-1}, preferably at least about 5 X 10^5 M^{-1} sec^{-1}, and most preferably at least about 7.5 X 10^5 M^{-1} sec^{-1}.

Because already available antibodies may lack sufficient potency to be advantageous for therapeutic and research uses, it is a still further object of the present invention to provide methods for increasing the potency of such antibodies by modifying said antibodies such that the k_{on} value exhibited by such antibody with respect to a given antigen will be increased preferably without changing the epitope to which the antibody binds.

It is a still further object of the present invention to provide a means of screening antibodies for properties that will insure high potency with respect to a desired antigen, said potency being at least 2- to 10-fold over known antibodies.

More specifically, it is an object of the present invention to produce antibodies having k_{on} values at least as high as 2.5 X 10^5 M^{-1} sec^{-1}, preferably at least 5 X 10^5 M^{-1} sec^{-1}, and most preferably as high as 7.5 X 10^5 M^{-1} sec^{-1}.

.

It is another object of the present invention to modify antibodies to increase the potency thereof by changing amino acids in the complementarity determining regions (CDRs) of such antibodies to increase the k_{on} value and thereby increase the potency thereof.

It is also an object of the present invention to provide high affinity, high potency antibodies having high specificity toward one or more antigens exhibited by an infectious microorganism (or microbe), especially one that causes infection of the respiratory system, most especially viruses.

In one embodiment, the present invention provides recombinant (including monoclonal) antibodies having substantially the variable chain framework (FR) regions of the antibody disclosed in Figure 1 (with the same specificity as this antibody) but wherein the antibodies of the present invention contain one or more amino acid differences in one or more of the CDRs (or complementarity determining regions) thereof. In a preferred embodiment, the antibodies of the present invention will differ from the antibody of Figures 1 or 2 (hereafter, the "basic antibody" or "reference antibody") only in the sequences of one or more of the CDRs, including L1, L2, L3, H1, H2 and H3.

It is a yet further object of the present invention to provide methods for the production of high potency monoclonal antibodies so as to afford increased efficacy for therapeutic uses, thereby permitting administration of greater effective dosages, especially antiviral dosages, as well as preparation of low concentration formulations and compositions and improving delivery by reducing dose frequency and allowing smaller injection volume.

It is another object of the present invention to provide compositions comprising the antibodies disclosed herein wherein said antibodies are suspended in a pharmacologically acceptable carrier, diluent or excipient.

It is a still further object of the present invention to provide methods of preventing and/or treating diseases, such as is caused by viruses, especially respiratory syncytial virus, comprising the administering to a patient at risk thereof, or afflicted therewith, of a therapeutically effective amount of a composition containing an antibody as disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the amino acid sequence of the light and heavy chain variable regions of a high affinity monoclonal antibody whose potency can be increased by the methods of the present invention. For reference purposes, this antibody is the MEDI-493 antibody sequence disclosed in Johnson et al, *J. Infect. Dis.*, **176**:1215-1224 (1997). Here, the CDR regions are underlined while non-underlined residues form the framework regions of the variable regions of each antibody. In this antibody, CDRs is derived from a mouse antibody while the framework regions are derived from a human antibody. The constant regions (not shown) are also derived from a human antibody. Figure 1A shows the light chain variable region and Figure 1B shows the heavy chain variable region of the light and heavy chains, respectively.

Figure 2 shows the heavy and light chain variable regions for a different basic or reference antibody sequence. Again, CDR regions are underlined. This sequence differs from Figure 1 in the first 4 residues of CDR L1 of the light chain, residue 103 of the light chain and residue 112 of the heavy chain. All of the high potency neutralizing antibodies, and active fragments thereof, of the present invention use the framework sequences of this reference or basic antibody. Fig. 2A shows the light chain and Fig. 2B shows the heavy chain.

Figure 3 shows the heavy and light chain variable regions of a preferred embodiment of the present invention. This preferred antibody has several high k_{on} CDRs present, which give rise to higher association rate constants (i.e., k_{on}) than the basic or reference antibody of Figure 2 and thus higher potency. This preferred antibody has the same framework amino acid sequences as the sequence of Figure 2 and, for purposes of the present disclosure, is denoted as "clone 15" in Tables 2 and 3, below. These

sequences are readily generated by the methods disclosed herein, all of which are readily known to those of skill in the art. The kinetic constants were measured according to the procedure of Example 1 and the potency determined as described in Example 2.

Figure 4 shows a schematic diagram of the use of phage M13 for generation of Fab fragments in accordance with the present invention and using a histidine six tag sequence to facilitate purification.

Figure 5 shows a schematic diagram for the screening procedure used for the antibodies of the present invention. "SPE" refers to a single point ELISA. "H3-3F4" is a designation for clone 4 of Tables 2 and 3.

DETAILED SUMMARY OF THE INVENTION

The present invention is directed to methods of producing high affinity neutralizing antibodies with high specificity and potency, or biological activity, preferably having an affinity of at least about 10^9 M^{-1}, and having a k_{on} value of at least about 2.5 x 10^5 M^{-1} sec^{-1}, preferably at least about 5 X 10^5 M^{-1} sec^{-1}, and most preferably at least about 7.5 x 10^5 M^{-1} s^{-1}.

With the advent of methods of molecular biology and recombinant DNA technology, it is now possible to produce such antibodies by recombinant means and thereby generate gene sequences that code for specific amino acid sequences found in the polypeptide structure of the antibodies. This has permitted the ready production of antibodies having sequences characteristic of neutralizing antibodies from different species and sources.

Regardless of the source of the antibodies, or how they are recombinantly constructed, all antibodies have a similar overall 3 dimensional structure. This structure is often given as L_2H_2 and refers to the fact that antibodies commonly comprise 2 light (L) amino acid chains and 2 heavy (H) amino acid chains. Both chains have regions capable of interacting with a structurally complementary antigenic target. The regions interacting with the target are referred to as "variable" or "V" regions and are characterized by differences in amino acid sequence from antibodies of different antigenic specificity.

The variable regions of either H or L chains contain the amino acid sequences capable of specifically binding to antigenic targets. Within these sequences are smaller sequences dubbed "hypervariable" because of their extreme variability between antibodies of differing specificity. Such hypervariable regions are also referred to as "complementarity determining regions" or "CDR" regions. These CDR regions account for the basic specificity of the antibody for a particular antigenic determinant structure.

The CDRs represent non-contiguous stretches of amino acids within the variable regions but, regardless of species, the positional locations of these critical amino acid sequences within the variable heavy and light chain regions have been found to have similar locations within the amino acid sequences of the variable chains. The variable heavy and light chains of all antibodies each have 3 CDR regions, each non-contiguous with the others (termed L1, L2, L3, H1, H2, H3) for the respective light and heavy chains. The accepted CDR regions have been described by Kabat et al, *J. Biol. Chem.* **252**:6609-6616 (1977). The numbering scheme is shown in the figures, where the CDRs are underlined and the numbers follow the Kabat scheme.

In all mammalian species, antibody polypeptides contain constant (i.e., highly conserved) and variable regions, and, within the latter, there are the CDRs and the so-called "framework regions" made up of amino acid sequences within the variable region of the heavy or light chain but outside the CDRs.

In accordance with the invention disclosed herein, such affinity constants are association constants and are measured by the kinetics of antigen-antibody complex formation, with the rate constant for association to form the complex being denoted as k_{on} and the dissociation constant being k_{off}. Measurement of such constants is well within the ordinary skill in the art and the details will only briefly be described further herein. The antibody and respective antigen combine to form a complex as follows:

Antibody (Ab) + Antigen (Ag) <----------> Ab-Ag

Such reaction can be described kinetically as a dynamic equilibrium:

$$Ab + Ag \underset{k_{off}}{\overset{k_{on}}{<----------->}} Ab\text{-}Ag$$

Thus, the k_{on} value is the rate constant, or specific reaction rate, of the forward, or complex-forming, reaction, as measured in units: M^{-1} sec $^{-1}$.

The values of k_{on} for the antibodies prepared by the methods disclosed herein were measured using the BIAcore protocol and equipment as disclosed in the Examples below.

In accordance with the foregoing, the present invention relates to high potency neutralizing antibodies, including immunologically active portions, fragments, or segments thereof, having a k_{on} of at least 2.5×10^5 M^{-1} s^{-1}, preferably at least about 5×10^5 M^{-1} s^{-1}, and most preferably at least about 7.5×10^5 M^{-1} s^{-1}.

As used herein, the terms "portion," "segment," and "fragment," when used in relation to polypeptides, refer to a continuous sequence of residues, such as amino acid residues, which sequence forms a subset of a larger sequence. For example, if a polypeptide were subjected to treatment with any of the common endopeptidases, such as trypsin, chymotrypsin, pepsin, papain, etc., the oligopeptides resulting from such treatment would represent portions, segments or fragments of the starting polypeptide. Such proteainases are commonly used to generate fragments of antibodies, such as those described herein, although such fragments can now more easily be generated by direct cloning or synthesis of the particular polypeptide desired to be produced. If used in relation to a polynucleotides, such terms refer to the products produced by treatment of said polynucleotides with any of the common endonucleases.

The antibodies of the present invention are high potency antibodies, generally exhibiting high k_{on} values (as defined elsewhere herein). For purposes of the present disclosure, the term "high potency" refers to a potency reflected by an EC_{50} (or effective concentration showing at least a reduction of 50% in the OD_{450} in the below described microneutralization assays) of below about 6 nM (nanomolar or 10^{-9} molar). The antibodies according to the present invention may be either neutralizing (causing destruction of the target species, such as a virus, and thus decreasing viral load) or may be non-neutralizing in nature and/or action and the use of the word "neutralizing" or lack of such use

is not limiting. Thus, an antibody not neutralizing for one use may be neutralizing for a different use.

The high potency antibodies of the present invention may have specificity for antigenic determinants found on microbes and are capable of neutralizing said microbes by attaching thereto. In accordance with the present invention, such microbes are most often viruses, bacteria or fungi, especially organisms that cause respiratory disease and most preferably viruses. A specific example, used in the examples herein, is respiratory syncytial virus (RSV); another example is parainfluenza virus (PIV).

The high potency antibodies of the present invention may also have specificity for antigens displayed on the surfaces of cancer cells (but will generally not include antibodies, such as vitaxin, which are anti-angiogenic in nature and thus work by a different mechanism. (See: Wu et al (1998))

The high potency antibodies of the present invention may also have specificity for chemical substances such as toxic substances, or toxins, or for the products of toxins, including, but not limited to, products produced by an organism's metabolism of such toxin(s). For example, the high potency antibodies of the present invention may be useful in nullifying, or otherwise ameliorating, the effects of addictive drugs, such as cocaine.

The high potency antibodies of the present invention may also have high affinity for their specific antigen, such as the F antigen of RSV, and, where such high affinity is exhibited, the affinity constant (K_a) of such antibodies is at least about 10^9 M^{-1}, preferably at least about 10^{10} M^{-1}, and most preferably at least about 10^{11} M^{-1}.

The high potency antibodies of the present invention exhibit high potency when measured in various assays of biological activity (see Methods below), such as the microneutralization assay described in Example 2 herein. Thus, the antibodies of the present invention show high potency as measured by the EC_{50} value measured using such assay and commonly have an EC_{50} of less than about 6.0 nM (nanomolar or 10^{-9} M), preferably less than about 3.0 nM, and most preferably less than about 1.0 nM.

The high potency antibodies of the present invention exhibit such high potency due to their high k_{on} values, which characteristic is derived from the nature of the amino acid sequences making up the framework (FR) and complementarity determining regions (CDRs) and thus comprise one or more high potency complementarity determining regions (CDR) within their amino acid sequences. Thus, the high potency neutralizing antibodies of the present invention may comprise at least 2 high potency CDRs, or 3 high potency CDRs, or even 4 high potency CDRs, or 5 high potency CDRs, and may even comprise 6 high potency CDRs. Of course, in the latter case, all 6 CDRs of the antibody, or active fragment thereof, are high potency CDRs. In accordance therewith, such high potency neutralizing antibodies of the present invention have high potency CDRs that consist of one each of light chain CDRs L1 (CDR L1), L2 (CDR L2), and L3 (CDR L3) and heavy chain CDRs H1 (CDR H1), H2 (CDR H2) and H3 (CDR H3).

In specific embodiments of such high potency antibodies, said high potency CDRs have amino acid sequences selected from the group consisting of SEQ ID NO: 11, 12, and 13 for CDR L1, SEQ ID NO: for CDR L1, SEQ ID NO: 14, 15, 16, 17, 18, 19, 20, 21, and 22 for CDR L2, SEQ ID NO: 23 for CDR L3, SEQ ID NO: 24 and 25 for CDR H1, SEQ ID NO: 26, 27, 28, 29, and 30 for CDR H2, SEQ ID NO: 31, 32, 33 and 34 for CDR H3.

In preferred embodiments, the high potency neutralizing antibodies of the present invention comprise variable heavy and light chains with amino acid sequences selected from the group consisting of SEQ ID NO:

The present invention further relates to a process for producing a high potency antibody comprising:

(a) producing a recombinant antibody, including immunologically active fragments thereof, comprising heavy and light chain constant regions derived from a mammalian antibody and heavy and light chain variable regions containing one or more framework and/or complementarity determining regions (CDRs) having preselected amino acid sequences;

(b) screening said recombinant antibodies for high association constant (k_{on}) when said antibody reacts *in vitro* with a selected antigen; and

(c) selecting antibodies with said high association constant (k_{on}).

The antibodies produced according to the present invention will commonly have high affinity and association constants, the latter commonly leading to high biological activity, or potency. In specific embodiments, the high potency antibodies produced according to the present invention commonly have an association constant (k_{on}) of at least about 2.5×10^5 M^{-1} s^{-1}, preferably at least about 5×10^5 M^{-1} s^{-1}, and most preferably at least about 5×10^5 M^{-1} s^{-1}.

In accordance with the processes disclosed herein, the preselected amino acid sequence producing a high k_{on} is present in either both framework region and at least one CDR region of the antibody or is limited only to the complementarity determining regions, of which there are commonly 6 per antibody molecule. Thus, in one embodiment, the process of the invention produces a high potency antibody wherein the preselected amino acid

sequence producing a high k_{on} is present in either both framework region and at least two or three CDR regions, perhaps all six CDR regions, of the antibody or is restricted to just CDR regions.

In another embodiment, the process of the invention produces a high potency antibody wherein the preselected amino acid sequence producing a high k_{on} is present in either both framework region and at least three CDR regions of the antibody or is restricted to just CDR regions.

In an additional embodiment, the process of the invention produces a high potency antibody wherein the preselected amino acid sequence producing a high k_{on} is present in either both framework region and at least four CDR regions of the antibody or is restricted to just CDR regions.

In addition, the antibodies produced according to the present invention may be complete tetrameric antibodies, having the H_2L_2 structure, or may be fragments of such antibody structures, including single chain antibodies or fragments such as Fab fragments.

In accordance with the present invention, the antigen for which the antibodies are specific are often, but not always, antigens expressed by viruses, such as respiratory syncytial virus (RSV) or parainfluenza virus (PIV).

The present invention also relates to a process for producing a high potency antibody comprising producing a recombinant antibody comprising heavy and light chain constant region derived from a mammalian antibody and heavy and light chain variable region containing framework and/or complementarity determining regions (CDR) wherein at least one CDR is a high k_{on} CDR having an amino acid sequence not found in nature and wherein the presence of said CDR results in a high k_{on}.

In specific embodiments, the processes of the present invention produce high potency recombinant antibodies wherein the recombinant high k_{on} antibody comprises at least two high k_{on} CDRs, possibly three high k_{on} CDRs, and even four high potency CDRs, and as many as five or six high potency CDRs.

In further embodiments of the methods of the invention, the aforementioned high association constant of the antibodies produced by the methods of the invention are at least about 2.5 x 10^5 M^{-1} s^{-1}, preferably at least about 5 X 10^5 M^{-1} s^{-1}, and most preferably at least about 5 x 10^5 M^{-1} s^{-1}.

The present invention further relates to a process for producing a high potency antibody comprising:

(a) producing a recombinant antibody, including immunologically active fragments thereof, comprising heavy and light chain constant regions derived from a mammalian antibody and heavy and light chain variable regions containing one or more framework and/or complementarity determining regions (CDRs) having preselected amino acid sequences;

(b) screening said recombinant antibodies for both high affinity and high association constant (k_{on}) when said antibody reacts *in vitro* with a selected antigen; and

(c) selecting antibodies with both high affinity and high association constant (k_{on}).

In a specific embodiment of this process, the antibodies produced thereby will have a high association kinetic constant (k_{on}), such as an association of at least about 2.5 x 10^5 M^{-1} s^{-1}, preferably at least about 5 X 10^5 M^{-1} s^{-1}, and most preferably at least about 7.5 x 10^5 M^{-1} s^{-1}.

The processes of the present invention also produce antibodies that may have both a high association constant for binding to a specific antigen as well as a high affinity constant for said antigen. In specific embodiments, said high affinity is reflected by high affinity constants of at least about 10^9 M^{-1}, preferably at least about 10^{10} M^{-1}, and most preferably at least about 10^{11} M^{-1}.

In preferred embodiments of the present invention, the processes disclosed herein produce high potency antibodies having both high affinity and high association constants wherein the high affinity constant is at least 10^9 M^{-1} and high association constant is at least 2.5×10^5 M^{-1} s^{-1}, especially where said high affinity is at least 10^{10} M^{-1} and said high k_{on} is at least 2.5×10^5 M^{-1} s^{-1}, most especially where said high affinity is at least 10^{11} M^{-1} and said high k_{on} is at least 2.5×10^5 M^{-1} s^{-1}, with most preferred embodiments having very high affinity and association constants, especially where said high affinity is at least 10^9 M^{-1} and said high association constant is at least 5×10^5 M^{-1} s^{-1}, and most especially where said high affinity is at least 10^{10} M^{-1} and said high association constant is at least 2.5×10^5 M^{-1} s^{-1}, the most especially preferred embodiment being one wherein the processes of the invention produce a high potency antibody wherein the affinity constant is at least 10^{11} M^{-1} and the association constant is at least 7.5×10^5 M^{-1} s^{-1}. It is to be understood that, where high affinity is also sought, any combination of the above mentioned affinity (K_a) and kinetic constants (k_{on}) are within the present invention.

These embodiments of the present invention also include processes wherein the preselected amino acid sequence producing a high k_{on} is present in either both framework region and at least one CDR region of the antibody or in at least one CDR region thereof, preferably either both framework and at least two CDRs thereof or in at least two CDRs thereof, most preferably

either both framework region and at least three, perhaps even four, CDRs, thereof or in at least three, or even four, five or six CDRs thereof, with no changes to the framework residues. Any changes to framework residues are merely for the purpose of making the CDR sequences fit better within the overall conformation and for interaction of said CDRs with the antigen of choice. Methods of doing this are well within the skill of those in the art and will not be discussed further herein. Utilizing such selected amino acid sequences in either both framework and CDR regions, or just CDR regions, where said sequences are present in as many as five, or even all six, CDRs represent alternative embodiments of the present invention.

The methods of the present invention are not limited to merely producing novel high affinity antibodies that are specific for a particular antigen and which have been produced without regard to already existing immunogenic molecules and structures. Thus, the methods disclosed herein find ample utility in their ability to provide suitable guidance in selected modifications to the structures of known antibody molecules thereby producing increases in the potency of an already known antibody, or active fragments thereof. Thus, the present invention also relates to a process for increasing the potency of an antibody comprising selectively changing one or more amino acids within the variable region framework and/or CDR regions of the antibody so as to increase the measured k_{on} value of said antibody.

Such substitutions or changes in the antibody amino acid sequence, or in the sequence of active fragments thereof, for example, in Fab fragments thereof, may occur anywhere in the heavy and light chain variable regions of said antibody, or active fragments thereof, including changes in both the framework region and one or more CDR regions, or such substitutions or changes may be restricted only to one or more of the CDR regions of the antibody, or active fragments thereof, whose potency is to be increased by

the methods disclosed according to the present invention. Thus, in one embodiment, the amino acid changes are restricted to the CDR portions of said variable regions.

In separate embodiments of the present invention, the antibody whose potency is to be increased will have an affinity constant of at least 10^9 M^{-1}, preferably at least about 10^{10} M^{-1}, and most preferably at least about 10^{11} M^{-1}.

In keeping with the disclosure herein, the antibodies produced according to the methods of the invention will have higher association constants after said amino acid changes and as a result of said amino acid changes, especially where the k_{on} value following said amino acid changes is at least 2.5 X 10^5 M^{-1} sec^{-1}, especially wherein the k_{on} value following said amino acid changes is at least 5 X 10^5 M^{-1} s^{-1}, and most especially at least about 7.5 X 10^5 M^{-1} s^{-1} each such embodiment including antibodies of any of the aforementioned affinity constants (K_a).

In applying the methods of the present invention it is to be understood that the aforementioned changes in amino acid sequence used to increase the potency of an antibody, or active fragments thereof, such as Fab fragments, or the use of selected amino acid sequences to produce high potency antibodies, or active high potency fragments thereof, produce said high potency, or said increases in potency, through the production of high association constants, or through an increase in such association constants, and that, while such changes may produce an antibody of high affinity, or of increased affinity, such is merely a by-product of the amino acid sequences selected for incorporation into said antibodies and which higher affinities may result arithmetically from the fact that the affinity constant is itself merely a ratio of association and dissociation constants, respectively, and

that high potencies of the antibodies produced according to the present methods are due to the value of the association constant and not to the numerical value of the affinity constant. For example, the use of selected amino acid sequences for incorporation into one or more CDRs of an antibody molecule may result in a sizeable increase in both kinetic association (k_{on}) and dissociation (k_{off}) constants and, if both are increased by the same factor, the result, in accordance with the teaching herein, is an increase in potency, or realization of a high potency but with no resulting increase in affinity constant (because the ratio of the k_{on} to k_{off} constants is the same). Conversely, also in accordance with the teaching of the present invention, where use of such preselected amino acid sequences within the CDRs of a high potency antibody, or for production of an antibody with increased potency, results in a decreased dissociation constant (k_{off}) and no increased association constant, or no high association constant, thereby producing an antibody of high, or increased, affinity (again, because of the increased ratio of association to dissociation constant) no high potency antibody would be expected to result. Thus, in accordance with the present invention it has been found that there is no change in potency where the k_{off} value alone changes (again, because the affinity constant is a ratio of k_{on} to k_{off}, where k_{off} changes but k_{on} remains constant, the affinity constant will change as well but no change in potency is realized).

In accordance with the present invention, any convenient method is available for measurement of the potency of the antibodies, or active fragments thereof, such as Fab fragments. One such convenient method used to measure potency of the antibodies prepared according to the methods disclosed herein is the cotton rat model, details of which are disclosed in the examples provided below. Another is the microneutralization assay (see Example 2).

Also in accordance with the present invention, there is provided a process for preventing or treating a disease comprising administering to a patient at risk of such disease, or afflicted with such disease, a therapeutically effective amount of a high potency antibody, or active fragment thereof, for example, an Fab fragment thereof, selected from the group consisting of the antibodies produced according to the methods disclosed herein. In a preferred embodiment, the disease is caused by a virus, especially wherein said virus is selected from the group consisting of respiratory syncytial virus and parainfluenza virus.

The highly biologically active (i.e., high potency) neutralizing antibodies of the present invention are achieved through genetically engineering appropriate antibody gene sequences, i.e., amino acid sequences, by arranging the appropriate nucleotide sequences and expressing these in a suitable cell line. Any desired nucleotide sequences can be produced using the method of codon based mutagenesis, as described, for example, in U.S. Pat. Nos. 5,264,563 and 5,523,388 (the disclosures of which are hereby incorporated by reference in their entirety). Such procedures permit the production of any and all frequencies of amino acid residues at any desired codon positions within an oligonucleotide. This can include completely random substitutions of any of the 20 amino acids at an desired position or in any specific subset of these. Alternatively, this process can be carried out so as to achieve a particular amino acid a desired location within an amino acid chain, such as the novel CDR sequences according to the invention. In sum, the appropriate nucleotide sequence to express any amino acid sequence desired can be readily achieved and using such procedures the novel CDR sequences of the present invention can be reproduced. This results in the ability to synthesize polypeptides, such as antibodies, with any desired amino acid sequences. For example, it is now possible to determine the amino acid sequences of any desired domains of

an antibody of choice and, optionally, to prepare homologous chains with one or more amino acids replaced by other desired amino acids, so as to give a range of substituted analogs.

In applying such methods, it is to be appreciated that due to the degeneracy of the genetic code, such methods as random oligonucleotide synthesis and partial degenerate oligonucleotide synthesis will incorporate redundancies for codons specifying a particular amino acid residue at a particular position, although such methods can be used to provide a master set of all possible amino acid sequences and screen these for optimal function as antibody structures or for other purposes. Such methods are described in Cwirla et al, *Proc. Natl. Acad. Sci.* **87**:6378-6382 (1990) and Devlin et al., *Science* **249**:404-406 (1990).

In accordance with the invention disclosed herein, enhanced antibody variants can be generated by combining in a single polypeptide structure one, two or more novel CDR sequences according to the invention, each shown to independently result in enhanced potency or biological activity. In this manner, several novel amino acids sequences can be combined into one antibody, in the same or different CDRs, to produce antibodies with desirable levels of biological activity. Such desirable levels will often result from producing antibodies whose k_{on} values are at least about 2.5×10^5 M^{-1} sec^{-1}. By way of non-limiting example, 3 such novel CDR sequences may be employed and the resulting antibodies screened for potency, or biological activity, using either the cotton rat protocol or the microneutralization protocol described herein, where the said antibody demonstrates high affinity for a particular antigenic structure, such as the F antigen of RSV. The overall result would thus be an iterative process of combining various single amino acid substitutions and screening the resulting antibodies for antigenic affinity and potency in a step by step manner, thereby insuring that potency is

increased without sacrifice of a desirably high, or at least minimum value for, affinity.

Using the novel sequences and methods of the present invention, such an approach would avoid the time and expense of generating and screening all possible permutations and combinations of antibody structure in an effort to find the antibody with the maximum efficacy. Conversely, complete randomization of a single 10 amino acid residue CDR would generate over 10 trillion variants, a number virtually impossible to screen.

This iterative method can be used to generate double and triple amino acid replacements in a stepwise process so as to narrow the search for antibodies having higher affinity.

Conversely, it must be recognized that not all locations within the sequences of the different antibody domains may be equal. Substitutions of any kind in a particular location may be helpful or detrimental. In addition, substitutions of certain kinds of amino acids at certain locations may likewise be a plus or a minus regarding affinity. For example, it may not be necessary to try all possible hydrophobic amino acids at a given position. It may be that any hydrophobic amino acid will do as well. Conversely, an acidic or basic amino acid at a given location may provide large swings in measured affinity. It is therefore necessary also to learn the "rules" of making such substitutions but the determination of such "rules" does not require the study of all possible combinations and substitutions – trends may become apparent after examining fewer than the maximum number of substitutions.

In accordance with the present invention, such rules determine the amino acid changes that must be made in the CDR regions of antibodies, or

the amino acid sequences that must be prepared in wholly novel and synthetic antibody polypeptides, so as to achieve high affinities. However, it has now been discovered that, while high affinity is often a property of antibodies useful in therapeutic applications, such antibodies do not always have a sufficient potency to afford practical utility in such uses.

As already described, affinity, whether formulated as an association or dissociation constant, is a measure of the ratio of the k_{on} and k_{off} constants. Thus, the ratio of k_{on} to k_{off} provides an affinity constant. For example, a k_{on} of 10^5 M^{-1} sec^{-1} and a k_{off} of 10^{-5} sec^{-1} would combine to give an affinity constant of 10^{10} M^{-1}. However, antibodies showing such high affinity may still lack the potency required to make them useful therapeutic agents. In accordance with the present invention, applicant has found that antibody potency is dependent on the value of the k_{on} rate for the antibody binding reaction. Thus, an antibody, regardless of the affinity of the antibody for the respective antigen, will exhibit an increase in its potency (i.e., the effect of such antibody when used for therapeutic purposes) where said antibody for a given affinity has a higher k_{on} value.

In accordance with the methods of the present invention, increased potency of an existing antibody, regardless of its antigen affinity, is achieved through selective changes to one or more of the amino acids present in one or more of the CDR regions of said antibody whereby said amino acid changes have the effect of producing an increase in the k_{on} rate for said antibody, preferably with an increase in antibody affinity. Higher potency can be achieved with a higher k_{on} value even if the affinity remains the same or is reduced somewhat. Such an antibody is most advantageously produced by synthesis of the required polypeptide chains via synthesis in suitably engineered cells having incorporated therein the appropriate nucleotide sequences coding for the required polypeptide chains containing the altered

CDR segments. Also in accordance with the methods of the present invention, a novel antibody having a desirable level of potency, or biological activity, can be prepared *de novo* by incorporation of selected amino acids at selected locations within the CDR regions of said antibody polypeptide chains using genetically engineered cells as described herein or wholly through chemical synthesis of the required polypeptide chains with subsequent formation of the necessary disulfide bonds.

In this regard, it should be kept clearly in mind that the antibodies produced according to the methods of the present invention may be antibodies possessing tetrameric, dimeric or monomeric structures. Thus, the term "antibody" as used herein includes whole tetrameric antibody molecules, as are commonly found in nature, as well as portions and fragments thereof, including L_2H_2, LH, Fab, $F(ab')_2$, and other fragments, the only requirement of such structures being that they retain biological activity as measured by the assays and protocols described herein.

In accordance with the foregoing, the antibodies of the present invention are high affinity monoclonal antibodies. Such antibodies, however, are monoclonal only in the sense that they may be derived from a clone of a single cell type. However, this is not meant to limit the origin of such antibodies to conventional hybridoma technology. Such antibodies may readily be produced in cells that commonly do not produce such antibodies, such as CHO or COS cells. In addition, such antibodies may be produced in other types of cells, especially mammalian and even plant cells, by genetically engineering such cells to express and assemble the polypeptide light and heavy chains forming the antibody product. In addition, such chains can be chemically synthesized but, since they would be specific for a given antigenic determinant, would still constitute "monoclonal" antibodies within the spirit in which that term is used. Thus, as used herein, the term

monoclonal antibody is intended to denote more the specificity and purity of the antibody molecules produced by the methods disclosed herein rather than the mere mechanism used for production of said antibodies.

Also as used herein, the term potency, as it applies to the high potency antibodies of the present invention, is intended to describe the dependency of the effect of the antibody, when utilized for its intended purpose, on the concentration of such antibody. Thus, potency as used herein means biological activity of the antibody produced by the methods of the present invention and with respect to a given antigen. For ascertaining the efficacy of the methods of the invention, such potency, or biological activity, or biological effect, is measured, for example, for an anti-RSV antibody, by either the cotton rat procedure or the microneutralization procedure, as described hereinbelow in the Methods section. Conversely, the affinity of an antibody for the antigen is simply a mathematical measure of the association or dissociation constant for the binding of the antibody to the respective antigen, where association and dissociation constants are reciprocals of each other.

In addition, the affinities of the antibodies produced according to the methods of the present invention will typically be in the range of 10^{10} M^{-1}. This range may, for example, be within 10-fold, higher or lower, of 10^{10} M^{-1} or be more than 10-fold greater than 10^{10} M^{-1} or may even be numerically equal to 10^{10} M^{-1}. In such cases, the affinity is denoted by an affinity constant, which is in the nature of a binding constant so as to give units of reciprocal molarity. As such, the affinity of the antibody for antigen is proportional to the value of this constant (i.e., the higher the constant, the greater the affinity). Such a constant is readily calculated from the rate constants for the association-dissociation reactions as measured by standard

kinetic methodology for antibody reactions (as already described and in Example 1).

In one embodiment, the antibodies produced according to the methods of the present invention (other than where the term "antibody" means an active portion, fragment or segment, all of which, for purposes of the present disclosure, are considered to be included within the meaning of the term antibody) will commonly comprise a mammalian, preferably a human, constant region and a variable region, said variable region comprising heavy and light chain framework regions and heavy and light chain CDRs, wherein the heavy and light chain framework regions have sequences characteristic of a mammalian antibody, preferably a human antibody, and wherein the CDR sequences are similar to those of an antibody of some species other than a human, preferably a mouse. Where the framework amino acid sequences are characteristic of those of a non-human, the latter is preferably a mouse.

In another embodiment, the antibody is a human antibody wherein the antibody has a k_{on} value as hereinabove described in order to provide for improved potency.

In addition, antibodies produced according to the present invention so as to have increased potency, or biological activity, will commonly bind the same epitope as prior to applying the methods disclosed herein to increase the k_{on} value. Thus, after applying the methods of the present invention, the antibody will have CDR sequences similar, but not identical, to the CDR sequences prior to application of the methods disclosed herein. Thus, after applying the methods of the invention, at least one of the CDRs of said antibody will contain amino acid substitutions, substitutions comprising the

replacement of one or more amino acids in the CDR regions by non-identical amino acids.

In keeping with the foregoing, and in order to better describe the sequences disclosed according to the invention with respect to a humanized antibody against RSV, a basic or starting sequence of light and heavy chain variable regions of an antibody, or fragment thereof, whose potency is to be increased, are shown in Figure 1A (light chain variable region – SEQ ID NO: 1) and Figure 1B (heavy chain variable region – SEQ ID NO: 2) or an Fab fragment of such an antibody (for example, the sequences of Figure 2a (light chain variable region – SEQ ID NO: 3) and Figure 2B (heavy chain variable region – SEQ ID NO: 4). Also in accordance with the invention, specific amino acids different from those of these starting sequences were generated by recombinant methods starting with prepared nucleotide sequences designed to generate said amino acid sequences when expressed in recombinant cells. The products of said cells are the monoclonal antibodies of the present invention.

In one embodiment of the present invention, potency is increased using a neutralizing antibody against respiratory syncytial virus (RSV) having an affinity constant of at least 10^9 M^{-1}, and preferably at least 10^{10} M^{-1} (for the F antigen thereof) by increasing the k_{on} value to at least 2.5×10^5 M^{-1} sec^{-1}. The amino acids present in the CDRs of such an Fab fragment are shown in Table 3 (for example, clone 5).

In general, the approach used to determine affinity and kinetic constants of antibodies before and after application of the methods of the invention to increase the k_{on} value, was to generate nucleotide sequences for the genes expressing the desired antibody chains (in accordance with the present invention) and insert these into vectors that were then used to

transform COS-1 cells by standard protocols. The cells were grown in wells and the supernatant sampled and measured for antigen binding using standard ELISA techniques. These polynucleotides were designed so as to provide one or more amino acid replacements in the CDRs that could then be screened for increased k_{on} values, with beneficial replacements (those yielding increased k_{on} values) being selectively combined for increased affinity. These are then subsequently screened for binding affinity for the respective antigen, such as the F antigen of RSV versus the basic or reference antibody, thereby determining that no serious change in affinity resulted from the increase in k_{on} values.

More specifically, the present invention relates to an isolated antibody comprising an affinity constant of at least 10^9 M^{-1}, preferably at least 10^{10} M^{-1} and most preferably at least 10^{11} M^{-1} and wherein the k_{on} value of such antibody is at least about 2.5×10^5 M^{-1} sec^{-1}, preferably at least about 5×10^5 M^{-1} sec^{-1}, and most preferably at least 2.5×10^5 M^{-1} sec^{-1} (including all combinations thereof).

Also in accordance with the present invention, such isolated antibody may be any kind of antibody already known or newly synthesized and novel. Thus, antibodies produced according to the methods of the present invention will include an antibody selected from the group consisting of any naturally occurring mammalian antibody, naturally occurring human antibodies, naturally occurring mouse antibodies, single chain antibodies, chimeric antibodies (having constant regions of an antibody of one species and variable regions of an antibody of a different species), CDR-grafted antibodies (having the CDR regions of an antibody of one species and the constant and, possibly, framework regions of an antibody of a different species), humanized antibodies (in which selected amino acids, of either the variable framework and/or CDR regions, have been altered so as to be similar

to a human antibody despite such sequences being largely derived from a different species, such as a mouse), preferably humanized mouse antibodies, altered mammalian, preferably mouse, most preferably human, antibodies (wherein selected amino acids of an existing antibody have been altered at some point in the polypeptide chain, commonly through the techniques of genetic engineering, to afford antibody structures only similar to the antibody structures on which they are based), and wholly synthetic novel antibodies, the latter not previously existing in nature.

The present invention also relates to methods of increasing the potency of one of the aforementioned types of antibodies (as previously described) comprising selectively changing the amino acids within the variable regions of the antibody so as to increase the measured k_{on} value of said antibody with respect to a particular antigen. Of course, the k_{on} value may be different for the same antibody following the same amino acid changes where the reaction is measured using a different antigen or antigenic determinant. However, in such cases, affinities are also likely to change as the identity of the antigenic determinant changes.

Also in accordance with the methods of the present invention the amino acid changes introduced into the sequences of the polypeptides of such antibodies are preferably restricted to the CDR portions of the variable regions of the antibodies although these could involve changes to the framework regions as well.

Although the most advantageous CDR sequences are commonly identified by screening clones of the antibodies whose potency is to be increased by the methods disclosed herein, once such high potency clones have been identified the resulting antibody is most advantageously produced thereafter through synthesis of the appropriate heavy and light polypeptide

chains within suitable animal or plant cells following introduction into such cells of suitable vectors containing the appropriate DNA sequences corresponding to the desired amino acid sequences, taking advantage of the genetic code to design the required nucleotide sequences. As a consequence of this approach, wholly novel antibodies with high potency can be produced at the outset using the amino acid sequence identities suggested by the methods of the present invention without the need to select already existing antibody sequences for modification. Thus, the methods disclosed herein facilitate the production of high potency antibodies of a completely novel structure as well as the synthesis of the sequences of already known antibodies but with selected amino acid changes, especially in the CDR regions of said antibodies, so as to deliberately increase the k_{on} values of such antibodies and thereby increase their potency without destroying affinity for the desired antigen.

In one embodiment of the methods of the present invention, an already existing antibody is modified to increase the potency thereof by increasing the k_{on} value. In a preferred embodiment, the antibody is one with high affinities, e.g., at least about 10^9 M^{-1} or 10^{10} M^{-1}. The antibody is synthesized, using clones or genetically engineered animal or plant cells, so as to introduce amino acid changes into the heavy and/or light polypeptide chains of said antibody, preferably where said antibody changes are introduced into the complementarity determining regions (CDRs) of said polypeptide chains, to increase the k_{on} value for binding of said antibody to a particular antigen with concomitant increase in the potency of the antibody. Thus, the methods of the present invention are advantageously utilized to produce an antibody molecule wherein the k_{on} value of said antibody following the amino acid changes to its sequence, preferably the variable regions of said sequence, most preferably the CDR portions of said variable regions, is higher than the k_{on} value exhibited by said antibody prior to said

amino acid changes when the k_{on} values are measured with respect to the same antigen.

In general, where the methods of the present invention are applied to known antibodies, the k_{on} of said antibodies will be increased by at least 2-fold, preferably at least 5-fold, and most preferably at least 10-fold. More specifically, the k_{on} value of said antibody is increased to at least about 2.5 X 10^5 M^{-1} sec^{-1}, preferably increased to at least 5 X 10^5 M^{-1} sec^{-1}, most preferably at least 7.5 X 10^5 M^{-1} sec^{-1}.

Because the methods disclosed herein are equally effective for designing novel recombinant high potency antibodies previously unknown in nature or in the art, the present invention also relates to a method of producing an antibody having a k_{on} value of at least 2.5 X 10^5 M^{-1} sec^{-1}, comprising preparing an antibody whose polypeptide sequences contain selected amino acids at selected locations, especially within the CDR sequences of the variable regions of the polypeptide chains of the antibody, and then screening said antibodies for antibodies having a k_{on} value of at least 2.5 X 10^5 M^{-1} sec^{-1}. In the same way, the present invention also relates to a method of producing an antibody having a k_{on} value of at least 5 X 10^5 M^{-1} sec^{-1} or even 7.5 X 10^5 M^{-1} sec^{-1}, comprising preparing an antibody whose polypeptide sequences contain selected amino acids at selected locations, especially within the CDR sequences of the variable regions of the polypeptide chains of the antibody, and then screening said antibodies for antibodies having a k_{on} value of at least 5 X 10^5 M^{-1} sec^{-1} or 7.5 X 10^5 M^{-1} sec^{-1}, respectively. The present invention is directed to the production of novel antibodies with high potency, or known antibodies with higher potency, by selective changes in the amino acid sequences thereof, which changes are selected so as to produce a higher k_{on} value, which may or may

not change the affinity of said antibody for the respective antigen, either up or down. In some specific embodiments, the affinity is also increased.

The methods of the present invention can be utilized for the production of antibodies with high potency, or antibodies of increased potency, having affinity for any desired antigen, although such antigen is preferably an antigen characteristic of a microorganism, such as a bacterium, virus, or fungus, preferably a virus (for example, respiratory syncytial virus (RSV)).

In another embodiment, the present invention relates to a method of preventing or treating a disease comprising administering to a patient at risk of such disease, or afflicted with such disease, of a therapeutically active amount of an antibody prepared by the methods disclosed herein. Thus, such antibody may be a completely novel antibody or a known and clinically useful antibody whose potency has been increased by application of the methods of the present invention. The disease prevented or treated by antibodies prepared by the methods disclosed herein may commonly be diseases caused by microorganisms, such as bacteria and viruses, preferably viruses and most preferably RSV.

The antibodies thus disclosed will also commonly have framework regions derived from a human antibody but, where not so derived, preferably from a mouse.

In generating the clones, the basic or reference antibody (heavy and light chain variable regions (CDRs plus Framework) shown in Figures 1 and 2) was used as the "template" for generating the novel CDR sequences of the antibodies of the present invention, the latter imparting higher k_{on} values. Standard approaches to characterizing and synthesizing the six CDR libraries

of single mutations were used (see Wu et al, *Proc. Natl. Acad. Sci.* **95**:6037-6042 (1998), the disclosure of which is hereby incorporated by reference in its entirety). The target CDR was first deleted for each of the libraries prior to annealing the nucleotides. For synthesis of the libraries, the CDRs of a reference antibody (see Figure 2) were defined as in Table 1. Codon based mutagenesis for oligonucleotide synthesis to yield the CDR sequences of the invention was employed (as described above).

Libraries were initially screened by capture lift to identify the highest affinity variants. Subsequently, these clones were further characterized using capture ELISA and by titration on immobilized antigen. Following such screening, the antibodies are then screened for their respective k_{on} values, the positive effects of which are then measured by determination of potency. Figures 4 and have show addition details on preparation and screening procedures used here.

Table 1. Basic CDR sequences as provided in Figure 2.

CDR	Residues of Fig. 2	Sequence	SEQ ID NO.
L1	24-33	**SA**SS**S**VGYMH	5
L2	49-55	DT**SKLA**S	6
L3	88-96	FQGS**G**YP**F**T	7
H1	31-37	T**S**GMSVG	8
H2	52-67	DIWWD**D**K**KD**YNPSLK**S**	9
H3	100-109	**S**MI**T**N**W**YFDV	10

In accordance with the present invention, DNA from the highest k_{on} variants is then sequenced to determine the nature of the beneficial or high potency replacements. After screening, antibodies are then prepared with the high-k_{on} amino acid replacements, either singly or in various combinations, so as to maximize the effects of such substitutions and thereby produce high affinity antibodies also exhibiting high potency.

As a general rule, the most beneficial of high k_{on} CDRs were found to result from amino acid replacements in up to 6 CDRs. Thus, the high potency (i.e., high k_{on}) neutralizing antibodies of the present invention contain amino acid sequences differing from that of the base or reference antibody (for example, as shown in Figures 1 and 2) only in complementarity determining regions L1 (or CDRL1), L2 (or CDRL2), L3 (or CDRL3), H1 (or CDRH1) and H3 (or CDRH3).

Thus, for the amino acid sequences of Figure 3, selected amino acids of the sequence of Figure 2 were replaced as a means of increasing the potency of the antibody with heavy and light chain sequences shown in Figure 2.

Selected high k_{on} antibodies (and active fragments thereof) resulting from the methods disclosed herein are shown in Table 2 (all of which have the framework sequences of Figure 2) where the reference clone is the clone with heavy and light chain variable region sequences shown in Figure 2 (SEQ ID NO: 3 and 4 for the light and heavy sequences, respectively).

Table 2. Sequences of CDRs tending to induce high potency in antibodies

Clone	CDR	High Potency CDR	Sequence	SEQ ID NO.
1	L1		SASSSVGYMH	5
	L2	X	DTFKLTS	14
	L3		FQGSGYPFT	7
	H1	X	TAGMSVG	24
	H2		DIWWDDKKDYNPSLKS	9
	H3	X	DMITNFYFDV	31
2	L1		SASSSVGYMH	5
	L2	X	DTFKLAS	15
	L3		FQGSGYPFT	7
	H1	X	TAGMSVG	24
	H2		DIWWDDKKDYNPSLKS	9
	H3	X	DMIFNWYFDV	32
3	L1		SASSSVGYMH	5
	L2	X	DTYKQTS	16
	L3		FQGSGYPFT	7
	H1	X	TAGMSVG	24
	H2		DIWWDDKKDYNPSLKS	9
	H3	X	DMIFNWYFDV	32
4	L1		SASSSVGYMH	5
	L2	X	DTRYLSS	17
	L3		FQGSGYPFT	7
	H1	X	TAGMSVG	24

		H2		DIWWDDKKDYNPSLKS	9
		H3	X	DMIFNWYFDV	32
	5	L1		SASSSVGYMH	5
5		L2	X	DTFKLAS	15
		L3		FQGSGYPFT	7
		H1	X	TAGMSVG	24
		H2		DIWWDDKKDYNPSLKS	9
		H3	X	DMITNFYFDV	31
10					
	6	L1		SASSSVGYMH	5
		L2	X	DTFKLAS	15
		L3	X	FQGSFYPFT	23
		H1	X	TAGMSVG	24
15		H2		DIWWDDKKDYNPSLKS	9
		H3	X	SMITNFYFDV	33
	7	L1	X	SASSRVGYMH	11
		L2	X	DTFKLAS	15
20		L3		FQGSGYPFT	7
		H1	X	TAGMSVG	24
		H2		DIWWDDKKDYNPSLKS	9
		H3	X	DMITNFYFDV	31
25	8	L1		SASSSVGYMH	5
		L2	X	DTFRLAS	16
		L3		FQGSGYPFT	7
		H1	X	TAGMSVG	24
		H2		DIWWDDKKDYNPSLKS	9
30		H3	X	DMITNFYFDV	31

	9	L1	X	SLSSRVGYMH	12
		L2	X	DTFYLSS	17
		L3		FQGSGYPFT	7
		H1	X	TPGMSVG	25
5		H2	X	DIWWDDKKHYNPSLKD	26
		H3	X	DMIFNFYFDV	31
	10	L1	X	SLSSRVGYMH	12
		L2	X	DTRGLPS	18
10		L3		FQGSGYPFT	7
		H1	X	TPGMSVG	25
		H2	X	DIWWDGKKHYNPSLKD	27
		H3	X	DMIFNFYFDV	31
15	11	L1	X	SPSSRVGYMH	13
		L2	X	DTMRLAS	19
		L3		FQGSGYPFT	7
		H1	X	TPGMSVG	25
		H2	X	DIWWDGKKHYNPSLKD	27
20		H3	X	DMIFNFYFDV	31
	12	L1	X	SLSSRVGYMH	12
		L2	X	DTFKLSS	20
		L3		FQGSGYPFT	7
25		H1	X	TAGMSVG	24
		H2	X	DIWWDGKKHYNPSLKD	27
		H3	X	DMIFNFYFDV	31

30

	13	L1	X	SASSRVGYMH	11
		L2	X	DTFKLSS	10
		L3		FQGSGYPFT	7
		H1	X	TAGMSVG	24
5		H2	X	DIWWDGKKDYNPSLKD	28
		H3	X	DMIFNFYFDV	34
	14	L1	X	SPSSRVGYMH	13
		L2	X	DTYRHSS	21
10		L3		FQGSGYPFT	7
		H1	X	TAGMSVG	24
		H2	X	DIWWDDKKHYNPSLKD	29
		H3	X	DMIFNWYFDV	32
15	15	L1	X	SLSSRVGYMH	12
		L2	X	DTMYQSS	22
		L3		FQGSGYPFT	7
		H1	X	TAGMSVG	24
		H2	X	DIWWDGKKSYNPSLKD	30
20		H3	X	DMIFNFYFDV	34
	16	L1		KCQLSVGYMH	13
		L2		DTSKLAS	6
		L3		FQGSGYPFT	7
25		H1		TSGMSVG	8
		H2		DIWWDDKKDYNPSLKS	9
		H3		SMITNWYFDV	10

30

	17	L1	SASSSVGYMH	5
		L2	DT**F**KLAS	15
		L3	FQGS**F**YPFT	23
		H1	T**A**GMSVG	24
5		H2	DIWWDDKKDYNPSLKS	9
		H3	SMITN**F**YFDV	33

10 Table 2 indicates the amino acid sequences (all sequences in standard amino acid one letter code) of the high k_{on} CDRs employed in the high potency antibodies prepared according to the methods disclosed herein. In table 2, the locations of key amino acid substitutions made in the corresponding CDRs of table 1 (i.e., locations at which CDRs differ in amino 15 acids) are indicated in bold face and underlined.

In accordance with the invention, by combining such amino acid substitutions so that more than one occurred in the same antibody molecule, it was possible to greatly increase the potency of the antibodies disclosed 20 herein.

In general, there is a correlation between k_{on} and potency of the antibody, with all of the higher k_{on} variants having more than one beneficial or high k_{on} CDR, including having all six CDRs substituted. .

25

In one embodiment, an antibody prepared so as to have increased k_{on} is an RSV-neutralizing antibody, with an affinity of at least 10^9 M^{-1} and preferably at least 10^{10} M^{-1}, that is also a humanized antibody that includes a human constant region and a framework for the heavy and light chains

wherein at least a portion of the framework is derived from a human antibody (or from a consensus sequence of a human antibody framework).

In another embodiment, all of the framework is derived from a human antibody (or a human consensus sequence).

In another embodiment, an antibody produced according to the present invention, with an affinity of at least 10^9 M^{-1} and preferably at least 10^{10} M^{-1}, is a grafted antibody having a human constant region, one or more CDRs that are derived from a non-human antibody in which at least one of the amino acids in at least one of said CDRs is changed and in which all or a portion of the framework is derived from a human antibody (or a consensus sequence of a human antibody framework).

Because the combination of CDR sequences of one antibody with non-CDR regions of another antibody results from a form of "grafting" of CDRs onto the remainder of the molecule, these have been referred to as "CDR grafted" antibodies. Today, using the techniques of genetic engineering the same product can be formed without isolating any sequences from actual antibodies. So long as the desired CDR sequences, and the constant and framework sequence are known, genes with the desired sequences can be assembled and, using a variety of vectors, inserted into appropriate cells for expression of the functional tetrameric antibody molecules. Coupling this with the methodology already described, permits the assembly of single mutation libraries wherein the antibodies possess the same sequences as corresponding grafted antibodies and, therefore, the same structure and binding affinities.

The relatively high k_{on} affinity antibodies of the invention can be present in a relatively pure or isolated form as well as in a supernatant drawn

from cells grown in wells or on plates. The antibodies of the invention can thus also be present in the form of a composition comprising the antibody of the invention and wherein said antibody is suspended in a pharmacologically acceptable diluent or excipient. The antibodies of the invention may be present in such a composition at a concentration, or in an amount, sufficient to be of therapeutic or pharmacological value in treating or preventing diseases, (for example, preventing RSV, including the higher incidence of asthma and weazing that often occur following such infections). Said antibodies may also be present in a composition in a more dilute form.

Consequently, the invention is also directed to providing a method of preventing and/or treating disease, especially viral diseases, most especially respiratory syncytial virus infections, comprising the administering to a patient at risk thereof, or afflicted therewith, of a therapeutically effective amount of the antibody composition described herein.

In one particular embodiment, a high potency neutralizing antibody of the present invention has the sequence of Figure 3 (SEQ ID NO: 101 and 102) for clone 15 with the CDRs of clone 15 given in Table 2).

It should be kept in mind that while the increased k_{on} antibodies of the present invention could be assembled from CDR regions and non-CDR regions derived from actual neutralizing antibodies by splicing amino acid segments together (and antibodies so assembled would be within the invention disclosed herein) the antibodies of the present invention are most conveniently prepared by genetically engineering appropriate gene sequences into vectors that may then be transfected into suitable cell lines for eventual expression of the assembled antibody molecules by the engineered cells. In fact, such recombinant procedures were employed to prepare the antibodies disclosed herein. In addition, because the sequences of the chains of the high affinity antibodies are known from the disclosure herein, such

antibodies could also be assembled by direct synthesis of the appropriate chains and then allowed to self-assemble into tetrameric antibody structures.

General Materials and Methods

Monoclonal Antibodies. MEDI-493 is an IgG_1 (COR)/ kappa (K102) humanized MAb (heavy and light chain variable region sequences shown in Figure 1) containing the antigen binding determinants of murine MAb 1129 [Johnson et al, *J. Infect. Dis.*, **176**, 1215-1224 (1997); Beeler and van Wyck Coelingh, *J. Virol.*, **63**, 2941-2950 (1989)].

RSV Fusion Inhibition Assay. The ability of the antibodies to block RSV-induced fusion after viral attachment to the cells was determined in a fusion inhibition assay. This assay was identical to the microneutralization assay, except that the cells were infected with RSV (Long) for four hours prior to addition of antibody [Taylor et al, *J. Gen. Virol.*, **73**, 2217-2223 (1992)]

BIAcore Analysis. Epitope analysis of the MAbs was performed using a BIAcore biosensor (BIAcore, Piscataway, NJ) [Karlsson et al, *J. Immunol. Methods*, **145**, 229-240 (1991); Johne, *Mol. Biotechnol.*, **9**, 65-71 (1998)] with a plasmin resonance microfluidics system. The antigen used for this assay was a truncated RSV (A2) F protein (amino acids 1-526) expressed in baculovirus. Purified RSV F protein was covalently coupled to an *N*-hydroxysuccinimide/l-ethyl-3-[3-dimethylaminopropyl]-carbodiimide - activated CM5 sensor chip according to the manufacturer's protocol, and unreacted active ester groups were reacted with 1 *M* ethanolamine. A primary injection of either 1 μM or 10 μM MEDI-493 was followed by an

HBSS wash step, and then by a secondary injection of either MEDI-493 or RHSZI9. Sensorgrams were analyzed using BIAevaluation software.

Isothermal Titration Calorimetry. The solution affinity of each MAb for RSV F protein was determined by isothermal titration calorimetry [Wiseman et al, *Anal. Biochem.*, **179**, 131-137 (1989)]. A 1.4 mL solution of 4.5 μ*M* RSV F protein was titrated with 5.5 μL injections of 26 μ*M* MEDI-493 or RSHZ19. After each injection of MAb, the amount heat given off, which is proportional to the amount of binding, was measured. The antigen used was an RSV (A2) F protein truncate (amino acids 25-524) expressed in drosophila cells. Titrations were conducted at 44° and 55°C to achieve optimal signal to noise. Thermal stability of the MAbs and the F protein at these temperatures was demonstrated by circular dichroism unfolding experiments. Affinities were corrected to 37°C for comparison with in vivo data using the integrated van't Hoff equation [Doyle and Hensley, *Methods Enzymol.*, **295**, 88-99 (1998)]. The van't Hoff correction is based solely on the F protein binding enthalpy change which was measured directly by calorimetry. Since the binding enthalpy changes for MEDI-493 and RSHZ19 were found to be very similar, the temperature corrections for their Kds were nearly identical.

Cotton Rat Prophylaxis. *In vivo* efficacy was determined using the cotton rat model [Prince et al, *J. Virol.*, **55**, 517-520 (1985)]. Cotton rats *(Sigmodon hispidus,* average weight 100 grams) were anesthetized with methoxyflurane, bled, and given 0.1 mL of purified MAb by intramuscular injection (i.m.) at doses of 5, 2.5, 1.25, or 0.625 mg/kg body weight, or bovine serum albumin (BSA) control at 5 mg/kg body weight. Twenty-four hours later animals were again anesthetized, bled for serum MAb concentration determination, and challenged by intranasal instillation (i.n.) of

10^5 PFU A (Long) or B (18537) strains of RSV. Four days later animals were sacrificed and their lungs were harvested. Lungs were homogenized in 10 parts (wt/vol) of Hanks balanced salt solution and the resultant suspension was used to determine pulmonary viral titers by plaque assay. Serum antibody titers at the time of challenge were determined by an anti-human IgG ELISA.

EXAMPLE 1

Kinetic Analysis of Humanized RSV Mabs by BIAcoreTM

The kinetics of interaction between high affinity anti-RSV Mabs and the RSV F protein was studied by surface plasmon resonance using a Pharmacia BIAcoreTM biosensor. A recombinant baculovirus expressing a C-terminal truncated F protein provided an abundant source of antigen for kinetic studies. The supernatant, which contained the secreted F protein, was enriched approximately 20-fold by successive chromatography on concanavalin A and Q-sepharose columns. The pooled fractions were dialyzed against 10 mM sodium citrate (pH 5.5), and concentrated to approximately 0.1 mg/ml. In a typical experiment, an aliquot of the F-protein (100 ml) was amine-coupled to the BIAcore sensor chip. The amount immobilized gave approximately 2000 response units (R_{max}) of signal when saturated with either H1129 or H1308F (prepared as in U.S. Patent 5,824,307, whose disclosure is hereby incorporated by reference). This indicated that there was an equal number of "A" and "C" antigenic sites on the F-protein preparation following the coupling procedure. Two unrelated irrelevant Mabs (RVFV 4D4 and CMV H758) showed no interaction with the immobilized F protein. A typical kinetic study involved the injection of 35 ml

of Mab at varying concentrations (25-300 nM) in PBS buffer containing 0.05% Tween-20 (PBS/Tween). The flow rate was maintained at 5 ml/min, giving a 7 min binding phase. Following the injection of Mab, the flow was exchanged with PBS/Tween buffer for 30 min for determining the rate of dissociation. The sensor chip was regenerated between cycles with a 2 min pulse of 10 mM HCl. The regeneration step caused a minimal loss of binding capacity of the immobilized F-protein (4% loss per cycle). This small decrease did not change the calculated values of the rate constants for binding and dissociation (also called the k_{on} and k_{off}, respectively).

More specifically, for measurement of k_{assoc} (or k_{on}), F protein was directly immobilized by the EDC/NHS method (EDC = N-ethyl-N'-[3-diethylaminopropyl)-carbodiimide). Briefly, 4 μg/ml of F protein in 10 mM NaoAc, pH 4.0 was prepared and about a 30 μl injection gives about 500 RU (response units) of immobilized F protein under the above referenced conditions. The blank flow cell (VnR immobilized-CM dextran surface) was subtracted for kinetic analysis. The column could be regenerated using 100 mM HCl (with 72 seconds of contact time being required for full regeneration). This treatment removed bound Fab completely without damaging the immobilized antigen and could be used for over 40 regenerations. For kon measurements, Fab concentrations were 12.5 nM, 25 nM, 50 nM, 100 nM, 200 nM, and 400 nM. The dissociation phase was analyzed from 230 seconds (30 seconds after start of the dissociation phase) to 900 seconds. Kinetics were analyzed by 1:1 Langmuir fitting (global fitting). Measurements were done in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Surfactant P20.

For measurements of combinatorial clones, as disclosed herein, the k_{on} and k_{off} were measured separately. The k_{on} was measured at conditions that

were the same as those for the single mutation clones and was analyzed similarly.

For measuring k_{dissoc} (or k_{off}), the following conditions were employed. Briefly, 4100 RU of F protein were immobilized (as above) with CM-dextran used as the blank. Here, 3000 RU of Fab was bound (with dissociated Fab high enough to offset machine fluctuation). HBS plus 5 nM F protein (about 350 - 2000 times higher than the K_{dissoc} or K_d - the dissociation equilibrium constant) was used as buffer. The dissociation phase was 6 - 15 hours at a flow rate of 5 µl/min. Under the conditions used herein, re-binding of the dissociated Fab was minimal. For further details, see the manual with the biosensor.

The binding of the high affinity anti-RSV antibodies to the F protein, or other epitopic sites on RSV, disclosed herein was calculated from the ratio of the first order rate constant for dissociation to the second order rate constant for binding or association ($K_d = k_{diss} / k_{assoc}$). The value for k_{assoc} was calculated based on the following rate equation:

$$dR/dt = k_{assoc}[Mab]R_{max} - (k_{assoc}[Mab] + k_{diss})R$$

where R and R_{max} are the response units at time t and infinity, respectively. A plot of dr/dt as a function of R gives a slope of (k_{assoc} [Mab] + k_{diss})--Since these slopes are linearly related to the [Mab], the value k_{assoc} can be derived from a replot of the slopes versus [Mab]. The slope of the new line is equal to k_{assoc}. Although the value of k_{diss} can be extrapolated from the Y-intercept, a more accurate value was determined by direct measurement of k_{diss}. Following the injection phase of the Mab, PBS/Tween buffer flows across the sensor chip. From this point, [Mab] = 0. The above stated equation for dR/dt thus reduces to:

$$dr/dt = k \quad \text{or} \quad dR/R = k_{diss}\, dt$$

Integration of this equation then gives:

$$\ln(R_0 / R_t) = k_{diss}\, t$$

where R_0 / R_t) are the response units at time 0 (start of dissociation phase) and t, respectively. Lastly, plotting $\ln(R_0 / R_t)$ as a function of t gives a slope of k_{diss}.

In the preferred embodiment herein, the numerical values from such antibody variants were as shown in Table 3:

Table 3. Summary of Kinetic Constants for High Potency Antibodies.

Clone No.	K_{on} x 10^5 ($M^{-1}s^{-1}$)	K_{on} x 10^{-4} (s^{-1})	EC_{50} (nM)
Ref.	1.85	6.5	3.52
1	3.65	3.26	2.26
2	5.31	4.22	5.05
3	6.05	4.22	4.70
4	7.57	4.62	3.55
5	4.16	3.06	2.61
6	1.85	3.20	2.88
7	3.70	2.51	1.59
8	3.75	2.73	2.67
9	6.63	2.82	0.29
10	5.27	2.99	1.06
11	5.71	7.17	20.9
12	7.9	4.53	3.24
13	7.43	2.30	0.81
14	7.35	2.50	2.23
15	7.81	2.80	0.56
16	2.04	7.35	6.12
17	1.09	2.49	2.7

For the clones in Tables 2 and 3, the reference clone is the Fab fragment with the sequences shown in Figure 2 and CDRs shown in Table 1. Clones 1-15 are Fab fragments having the framework sequences of Figure 2 and the indicated CDR sequences. The "X" indicates a high potency CDR (i.e., a CDR whose presence versus the reference sequence results in high potency (and higher potency than the reference Fab). Where no "X" appears

next to the CDR, the sequence is just the corresponding sequence of the reference Fab (from Table 1). Clones 16 and 17 are actual monoclonal antibodies with the framework sequences of Figure 1 and constant regions as described in Johnson et al (1997). The framework sequences of these antibodies may differ slightly from those of the Fab fragments.

Here, the amino acid sequences of the indicates CDRs represent the amino acid residues located at the key locations within the CDRs of the high potency antibodies produced by the methods of the present invention. For example, to increase the potency of an antibody by producing a higher k_{on} value, the amino acids located at the key positions as taught herein by the bold and underlined residues in Table 1 for the reference antibody would be replaced by the amino acids listed under CDRs in Table 2 (and also bold and underlined). Thus, these one letter codes represent the amino acids replacing the reference amino acids at the key positions (or critical positions) of the CDRs shown in Figure 2 (residues in bold in the sequences of Table 2) for a reference antibody whose potency is to be increased.

EXAMPLE 2

Microneutralization Assay

Neutralization of the antibodies of the present invention were determined by microneutralization assay. This microneutralization assay is a modification of the procedures described by Anderson et al ["Microneutralization test for respiratory syncytial virus based on an enzyme immunoassay, *J. Clin. Microbiol.* **22**, 1050-1052 (1985), the disclosure of which is hereby incorporated by reference in its entirety]. The procedure

used here is described in Johnson et al [*J. Infectious Diseases*, **180**, 35-40 (1999), the disclosure of which is hereby incorporated by reference in its entirety]. Antibody dilutions were made in triplicate using a 96-well plate. Ten $TCID_{50}$ of respiratory syncytial virus (RSV – Long strain) were incubated with serial dilutions of the antibody (or Fabs) to be tested for 2 hours at 37°C in the wells of a 96-well plate. RSV susceptible HEp-2 cells (2.5×10^4) were then added to each well and cultured for 5 days at 37°C in 5% CO_2. After 5 days, the medium was aspirated and cells were washed and fixed to the plates with 80% methanol and 20% PBS. RSV replication was then determined by F protein expression. Fixed cells were incubated with a biotin-conjugated anti-F protein monoclonal antibody (pan F protein, C-site-specific MAb 133-1H) washed and horseradish peroxidase conjugated avidin was added to the wells. The wells were washed again and turnover of substrate TMB (thionitrobenzoic acid) was measured at 450 nm. The neutralizing titer was expressed as the antibody concentration that caused at least 50% reduction in absorbency at 450 nm (the OD_{450}) from virus-only control cells.

WHAT IS CLAIMED IS:

1. A high potency antibody, including immunologically active portions, fragments, or segments thereof, other than vitaxin and having a k_{on} of at least 2.5×10^5 M^{-1} s^{-1}.

2. The high potency antibody of claim 1 wherein said k_{on} is at least about 5×10^5 M^{-1} s^{-1}.

3. The high potency antibody of claim 1 wherein said k_{on} is at least about 7.5×10^5 M^{-1} s^{-1}.

4. The high potency antibody of claim 1 wherein said antibody is a neutralizing antibody.

5. The high potency neutralizing antibody of claim 4 wherein said antibody has specificity for antigenic determinants found on microbes.

6. The high potency neutralizing antibody of claim 5 wherein said microbe is selected from the group consisting of viruses, bacteria and fungi.

7. The high potency neutralizing antibody of claim 5 wherein said microbe is a virus.

8. The high potency neutralizing antibody of claim 7 wherein said virus is selected from the group respiratory syncytial virus (RSV) and parainfluenza virus (PIV).

9. The high potency neutralizing antibody of claim 1 wherein said antibody is specific for antigens found on cancer cells.

9. The high potency neutralizing antibody of claim 1 wherein said antibody has an affinity constant (K_a) of at least about 10^9 M^{-1}.

10. The high potency antibody of claim 1 wherein said antibody is specific for a toxic substance or a product of a toxic substance.

11. The high potency neutralizing antibody of claim 1 wherein said antibody has an affinity constant (K_a) of at least about 10^9 M^{-1}.

12. The high potency neutralizing antibody of claim 1 wherein said antibody has an affinity constant (K_a) of at least about 10^{10} M^{-1}.

13. The high potency neutralizing antibody of claim 1 wherein said antibody has an affinity constant (K_a) of at least about 10^{11} M^{-1}.

14. The high potency neutralizing antibody of claim 1 wherein said antibody has an EC_{50} of less than 6.0 nM.

15. The high potency neutralizing antibody of claim 1 wherein said antibody has an EC_{50} of less than 3.0 nM.

16. The high potency neutralizing antibody of claim 1 wherein said antibody has an EC_{50} of less than 1.0 nM.

17. The high potency neutralizing antibody of claim 1, wherein said antibody comprises one or more high potency complementarity determining regions (CDR).

18. The high potency neutralizing antibody of claim 17, wherein said antibody comprises at least 2 high potency CDRs.

19. The high potency neutralizing antibody of claim 18, wherein said antibody comprises at least 4 high potency CDRs.

20. The high potency neutralizing antibody of claim 19, wherein said antibody comprises 6 high potency CDRs.

21. The high potency neutralizing antibody of claim 19, wherein said high potency CDRs consist of one each of light chain CDRs L1 (CDR L1), L2 (CDR L2), and L3 (CDR L3) and heavy chain CDRs H1 (CDR H1), H2 (CDR H2) and H3 (CDR H3).

22. The high potency neutralizing antibody of claim 17, wherein said high potency CDRs have amino acid sequences selected from the group consisting of SEQ ID NO: 11, 12, and 13 for CDR L1, SEQ ID NO: for CDR L1, SEQ ID NO: 14, 15, 16, 17, 18, 19, 20, 21, and 22 for CDR L2, SEQ ID NO: 23 for CDR L3, SEQ ID NO: 24 and 25 for CDR H1, SEQ ID NO: 26, 27, 28, 29, and 30 for CDR H2, SEQ ID NO: 31, 32, 33 and 34 for CDR H3.

23. The high potency neutralizing antibody of claim 1 wherein said antibody has an amino acid sequence selected from the group consisting of SEQ ID NO:

24. A process for producing a high potency neutralizing antibody comprising:

(a) producing a recombinant antibody, including immunologically active fragments thereof, comprising heavy and light chain variable regions

containing one or more framework and/or complementarity determining regions (CDRs) having preselected amino acid sequences;

(b) screening said recombinant antibodies for high association kinetic constant (k_{on}) when said antibody reacts *in vitro* with a selected antigen; and

(c) selecting antibodies with said high association kinetic constant (k_{on}).

25. The process of claim 24 wherein said k_{on} is at least 3×10^5 M^{-1} s^{-1}.

26. The process of claim 24 wherein said k_{on} is at least 10^6 M^{-1} s^{-1}.

27. The process of claim 24 wherein the preselected amino acid sequence producing a high k_{on} is present in both framework region and at least one CDR region of the antibody.

28. The process of claim 24 wherein the preselected amino acid sequence producing a high k_{on} is present in both framework region and at least two CDR regions of the antibody.

29. The process of claim 24 wherein the preselected amino acid sequence producing a high k_{on} is present in both framework region and at least four CDR regions of the antibody.

30. The process of claim 24 wherein the preselected amino acid sequence producing a high k_{on} is present in both framework region and six CDR regions of the antibody.

31. The process of claim 24 wherein said antibody is further screened in step (b) for an affinity constant of at least 10^9 M^{-1}.

32. The process of claim 24 wherein said antibody is further screened in step (b) for an affinity constant of at least 10^{10} M^{-1}.

33. The process of claim 24 wherein said antibody is further screened in step (b) for an affinity constant of at least 10^{11} M^{-1}.

34. The process of claim 24 wherein said high affinity constant is at least 10^{10} M^{-1} and said high association constant is at least 5 X 10^5 M^{-1} s^{-1}.

35. A process for producing a high potency neutralizing antibody comprising producing a recombinant antibody comprising heavy and light chain variable regions containing framework and/or complementarity determining regions (CDR) wherein at least one CDR is a high k_{on} CDR and wherein the presence of said CDR results in a high k_{on}.

36. The process of claim 35 wherein said recombinant high k_{on} antibody comprises at least two high k_{on} CDRs.

37. The process of claim 35 wherein said recombinant high k_{on} antibody comprises at least four high k_{on} CDRs.

38. The process of claim 35 wherein said recombinant high k_{on} antibody comprises six high k_{on} CDRs and wherein said high potency CDRs consist of one each of light chain CDRs L1 (CDR L1), L2 (CDR L2), and L3 (CDR L3) and heavy chain CDRs H1 (CDR H1), H2 (CDR H2) and H3 (CDR H3).

39. The process of claim 35 wherein said k_{on} is at least 5 X 10^5 M^{-1} s^{-1}.

40. The process of claim 35 wherein said k_{on} is at least 7.5×10^5 M^{-1} s^{-1}.

41. The process of claim 35 wherein said antibody also has an affinity constant (K_a) of at least 10^9 M^{-1}.

42. The process of claim 35 wherein said antibody also has an affinity constant (K_a) of at least 10^{10} M^{-1}.

43. The process of claim 35 wherein said antibody also has an affinity constant (K_a) of at least 10^{11} M^{-1}.

44. A process for increasing the potency of an antibody comprising selectively changing one or more amino acids within the variable region framework and/or CDR regions of the antibody so as to increase the measured k_{on} value of said antibody.

45. The process of claim 44 wherein the amino acid changes are restricted to the CDR portions of said variable regions.

46. The process of claim 44 wherein the affinity of said antibody prior to said amino acid changes is at least 10^9 M^{-1}.

47. The process of claim 44 wherein the affinity of said antibody prior to said amino acid changes is at least 10^{10} M^{-1}.

48. The process of claim 44 wherein the affinity of said antibody prior to said amino acid changes is at least 10^{11} M^{-1}.

49. The process of claim 44 wherein the k_{on} value following said amino acid changes is at least 5 X 10^5 M^{-1} sec^{-1}.

50. The process of claim 44 wherein the k_{on} value following said amino acid changes is at least 10^6 M^{-1} sec^{-1}.

51. A process for preventing or treating a disease comprising administering to a patient at risk of such disease, or afflicted with such disease, a therapeutically effective amount of an antibody, or fragment thereof, selected from the group consisting of the antibodies of claims 1, 24, 35 and 44.

52. The process of claim 51 wherein the disease is caused by a virus.

53. The process of claim 52 wherein said virus is selected from the group consisting of respiratory syncytial virus and parainfluenza virus.

54. The process of claim 51 wherein the antibody, or active fragment thereof, has a light chain variable region having the amino acid sequence of SEQ ID NO. and the heavy chain variable region has the amino acid sequence of SEQ ID NO.

55. The process of claim 51 wherein said antibody is an Fab fragment.

ABSTRACT

High potency antibodies, including immunologically active fragments thereof, having high kinetic association constants and optional high affinities are disclosed, along with methods for producing such antibodies. The high potency antibodies disclosed herein are of either the neutralizing or non-neutralizing type and have specificity for antigens displayed by microorganisms, especially viruses, as well as antigenic sites present on cancer cells and on various types of toxins, and the products of toxins. Processes for producing high potency neutralizing antibodies having selected amino acid sequences in their framework and/or complementarity determining regions (CDR) are also disclosed along with methods for increasing the potency of already existing neutralizing antibodies. Methods of using said antibodies in the prevention and/or treatment of diseases, especially diseases induced or caused by viruses, are also within the invention herein.

FIGURE 1

A

DIQMTQSPST LSASVGDRVT ITC<u>KCQLSVGYMH</u> WYQQKPG 40
CDR L1

KAPKLLIY <u>DTSKLAS</u> GVPSR FSGSGSGTEF TLTISSLQPD 80
CDR L2

DFATYYC <u>FQGSGYPFT</u> FGGGTKLEIK 106
CDR L3

B

QVTLRESGPA LVKPTQTLTL TCTFSGFSLS <u>TSGMSVG</u> WIR 40
CDR H1

QPPGKALEWL A <u>DIWWDDKKDYNPSLKS</u> RLT ISKDTSKNQV 80
CDR H2

VLKVTNMDPA DTATYYCAR <u>SMITNWYFDV</u> W GAGTTVTVSS 120
CDR H3

FIGURE 2

A

DIQMTQSPST LSASVGDRVT ITC<u>SASSSVGYMH</u> WYQQKPG 40
CDR L1

KAPKLLIY <u>DTSKLAS</u> GVPSR FSGSGSGTEF TLTISSLQPD 80
CDR L2

DFATYYC <u>FQGSGYPFT</u> FGGG TKVEIK 106
CDR L3

B

QVTLRESGPA LVKPTQTLTL TCTFSGFSLS <u>TSGMSVG</u> WIR 40
CDR H1

QPPGKALEWL A <u>DIWWDDKKDYNPSLKS</u> RLT ISKDTSKNQV 80
CDR H2

VLKVTNMDPA DTATYYCAR <u>SMITNWYFDV</u> WGQGTTVTVSS 120
CDR H3

2/5

FIGURE 3

A

DIQMTQSPST LSASVGDRVT ITCSLSSRVGYMH WYQQKPG 40
CDR L1

KAPKLLIY DTMYQSS GVPSR FSGSGSGTEF TLTISSLQPD 80
CDR L2

DFATYYC FQGSGYPFT FGGG TKVEIK 106
CDR L3

B

QVTLRESGPA LVKPTQTLTL TCTFSGFSLS TAGMSVG WIR 40
CDR H1

QPPGKALEWL A DIWWDGKKSYNPSLKD RLT ISKDTSKNQV 80
CDR H2

VLKVTNMDPA DTATYYCAR DMIFNFYFDV WGQGTTVTVSS 120
CDR H3

3/5

Fig. 4

M13 Phage Expression of Fab